REMARKS

Claims 293, 294 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point and distinctly claim the subject matter which applicant regards as the invention. The Examiner states the following:

> Claim 293 recites the limitation "wherein at least 2 consecutive peptide bonds...". There is insufficient antecedent basis for this limitation in the claim.

Applicants have amended Claim 293 to depend on Claim 292, and not on claim 287, to correct a typographical error, and provide sufficient antecedent basis.

Claims 287-289 stand rejected under 35 U.S.C. §102(b) as being anticipated over Ernst et al. (Cytometry 10:3-10 1989). The Examiner states the following:

> Ernst et al. discloses cyanine dye synthesis, comprising a first intermediate (III), a second intermediate 2,3,3,-Trimethyl(3H)-indole (page 5, 3rd paragraph) and a linking reagent which is first linked to the second intermediate to form compound V. The first intermediate comprises a reactive group capable a forming carbon-carbon linkage with a target. The reactive group comprises a halogenated compound.

Claims 287-289 stand rejected under 35 U.S.C. 102(b) as being anticipated by Randall et al. (US 6,114,350). The Examiner states the following:

> Randall et al. disclose cyanine dye synthesis, comprising a first intermediate, a second intermediate both of formula (I) and a linking reagent. (col. 5, 6). The first and second intermediates can comprise a reactive group capable of

> forming a carbon-carbon linkage with a target (see col. 2-col.3, bridging paragraph, and col. 6-col.8)). The reactive group can comprise a halogenated compound (col. 8, lines 14-16).

Applicants have amended Claim 287 to include the three limitations provided in Claim 288 (which has been canceled). Specifically, Claim 287 was amended to reflect that the reactive group may comprise an alkene group, an alkyne group, or a metallo-organic compound. Applicants purposely deleted the "halogenated compound" limitation since the Randall and Ernst references disclose halogens as reactive groups, yet they do not disclose alkene or alkyne groups, or metallo-organic compounds.

Claims 287-289 stand rejected under 35 U.S.C. 102(e) as being anticipated by Singh et al. (6,403,807). The Examiner states the following:

> Singh et al. disclose synthesis of cyanine dyes. Figure 1 shows a reaction scheme, illustrating synthesis of a cyanine dye by linking together two indolenine precursor units using linking reagents. Wherein in said units X1 is C; at least one of R1 through R10 comprises a reactive group capable of forming carbon-carbon linkages with a target. Said reaction results in the synthesis of a cyanine dye where n=2.

Applicants respectfully traverse this rejection. Singh does not disclose a reactive group that forms a carbon-carbon bond in the dyes. While their bridged dyes do form carbon-carbon bonds (as shown on the bottom of page 1, in Figure 2 and at the top of page 3, in Figure 2), if there are groups that are used to form carbon-carbon bonds to form this "bridge", they are lost prior to forming the $(=)_n$ linkage. Thus, Singh does not describe the dye shown in step (b) that has a reactive group that can form a carbon-carbon bond. Furthermore, the structure of the dye formed in step (b) of Claim 287 does not describe a chemical that joins R_1 to R_2 (as shown in Singh) and the dye formed in step (b) of Claim 287 only shows the joining of the two portions through the

SUMMARY

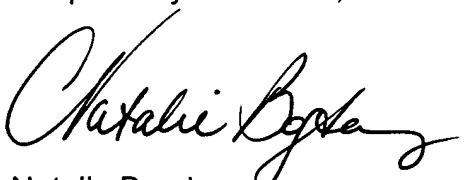
In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of record of Claims 287-289 and the objection of record of Claims 290-292 and 295-300. Therefore, Claims 287 and 288 to 300 are presented for further examination.

Early and favorable action is respectfully requested.

No other fee or fees are believed due in connection with this paper. In the event that any fee or fees are due, however, the United States Patent and Trademark Office is hereby authorized to charge any such fee or fees to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney requests that she be contacted at the number provided below.

Respectfully submitted,



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al/USProsecution/Enz-61(D1).amendment.021607

methine $(=)_n$ linkage. The only indication in Singh of the formation of a carbon-carbon bond is when the two dye portions join together through the methine bridge, however this site is not part of R_1 to R_{10} of the present invention.

Claims 290-292 and Claims 295-300 stand objected as being dependent upon a rejected base claim.

Since Applicants have amended Claim 287 to place it in what they believe is an allowable condition, Claims 290-292 and 295-300 no longer depend on a rejected claim.

Applicants also hereby submit the references (attached herein as Exhibit 3) which were listed in PTO-1449, filed on March 20, 2004, which were missing a date or not included in the filing.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/761,906	01/21/2004	Jannis G. Stavrianopoulos	Enz-61 (D1)	4378

28171 7590 08/21/2006

ENZO BIOCHEM, INC.
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EXAMINER	
RILEY, JEZIA	

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 08/21/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/761,906	STAVRIANOPOULOS ET AL.	
	Examiner	Art Unit	
	Jezia Riley	1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *287-300* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *287,289,293 and 294* is/are rejected.

7)☒ Claim(s) *288, 290-292, 295-300* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date 3/10/04
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Claim Rejections - 35 USC § 112

1. The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 293, 294 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 293 recites the limitation "wherein at least 2 consecutive peptide bonds ...". There is insufficient antecedent basis for this limitation in the claim.

Claim Rejections - 35 USC § 102

2. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

3. Claims 287-289 are rejected under 35 U.S.C. 102(b) as being anticipated by Ernst et al. (Cytometry 10:3-10 1989).

Ernst et al disclose cyanine dye synthesis, comprising a first intermediate (III), a second intermediate 2,3,3,-Trimethyl(3H)-indole (page 5, 3rd paragraph) and a linking reagent which is first linked to the second intermediate to form compound V. The first intermediate comprises a reactive group capable a forming carbon-carbon linkage with a target. The reactive group comprises a halogenated compound.

4. Claims 287-289 are rejected under 35 U.S.C. 102(b) as being anticipated by Randall et al . (US6114350).

Randall et al disclose cyanine dye synthesis, comprising a first intermediate , a second intermediate both of formula (I) and a linking reagent.(col. 5, 6). The first and second intermediates can comprise a reactive group capable a forming carbon-carbon linkage with a target (see col 2-col.3, bridging paragraph, and col. 6 –col.8)). The reactive group can comprise a halogenated compound (col. 8,lines 14-16).

5. Claims 287, 289 are rejected under 35 U.S.C. 102(e) as being anticipated by Singh et al. (6,403,807)

Singh et al. disclose synthesis of cyanine dyes.

Figure 1 shows a reaction scheme, illustrating synthesis of a cyanine dye by linking together two indolenine precursor units using linking reagents. Wherein in said units X1 is C; at least one of R1 through R10 comprises a reactive group capable of forming carbon-carbon linkages with a target. Said reaction results in the synthesis of a cyanine dye where n=2.

6. Claims 290-292, 295-300 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

7. The references lined through in the PTO 1449 filed 3/20/04, were either missing a date or were not in the file.

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jezia Riley whose telephone number is 571-272-0786. The examiner can normally be reached on 9:30AM - 5:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Thursday, August 17, 2006

JEZIA RILEY
PRIMARY EXAMINER

Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)	Atty. Docket No. ENZ-61(D1)	Serial No. 10/761,906
	Applicants: Ilan, et al	
	Filed: January 22, 2004	Group: Not yet known

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE
JR		5,494,810		Barany, et al			
JR		6,004,286		Bellhouse, et al			
JR		5,582,984		Bieniarz, et al			
JR		5,599,323		Bieniarz, et al			
JR		4,978,614		Bronstein, IY			
		5,462,854		Coassin, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANSLATION YES	TRANSLATION NO
JR	EP	0 667 393	8/16/95	Rabbani et al	A1			
JR	EP	0 070 685	7/14/82	Heller, et al	A1			

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

JR		Ball, et al., "The use of tailed octamer primers for cycle sequencing," Nucl. Acids. Res. 26:5225-5227 (1998)
JR		Baranov, et al., "A new technique for the characterization of long-range tertiary contacts in large RNA molecules: insertion of a photolabel at a selected position in 16S rRNA within the Escherichia coli ribosome," Nucl. Acids Res. 25:2266-2273 (1997)
		~~Dale, R.M., et al., "Direct covalent mercuration of nucleotides and polynucleotides," Biochemistry 14:2447-2457 (1975)~~
JR		Dale, R.M., et al., "The synthesis and enzymatic polymerization of nucleotide containing mercury: potential tools for nucleic acid sequencing and structural analysis," Proc. Natl. Acad. Sci. USA 70:2238-2242 (1973)
JR		Doan, T.L., et al., "Targeted cleavage of polynucleotides by complementary oligonucleotides covalently linked to iron-porphyrins," Biochemistry 25:6736-6739 (1986)
JR		Eglinton, G., et al., "A coupling of acetylenic compounds," Adv. Organic Synthesis 4:225-328 (1963)

EXAMINER	/Jezia Riley/	DATE CONSIDERED	08/17/2006

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE
JR		5 8 4 9 4 8 0		Cros, et al			
JR		4 8 9 4 3 2 5		Engelhardt, et al			
JR		5 2 4 1 0 6 0		Engelhardt, et al			
JR		5 2 8 8 6 0 9		Engelhardt, et al			
JR		6 2 2 1 5 8 1		Engelhardt, et al			
JR		4 3 7 5 9 7 2		Forgione, et al			
JR		5 2 1 0 0 1 5		Gelfand, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANSLATION YES	TRANSLATION NO
JR	WO	9 9 2 8 5 0 0	11/27/98	Lee, et al				
JR	EP	0 9 1 7 0 3 9	1/12/00	Rabbani, et al	A1			

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

		~~Enzo Biochem, Catalog Nos. 42722, 4723, 4724, New York, NY~~ No date
JR		Ernst, et al., "Cyanine dye labeling reagents for sulfhydryl groups," Cytometry 10:3-10 (1989)
JR		Fuhrop, J.H., et al., Chapter 19 in "Porphyrins and Metalloporphyrins," ed. Smith, K.M., Elsevier Science, New York (1975)
JR		Kawase, et al., "Studies on nucleic acid interactions. I. Stabilities of mini-duplexes (dG2A4XA4G2-dC2T4YT4C2) and self-complementary d(GGGAAXYTTCCC) containing deoxyinosine and other mismatched bases," Nucl. Acids. Res. 14:7727-7736 (1986)
JR		Kuhlmann, K.F., et al., "Synthesis, DNA-binding and biological activity of a double intercalating analog of ethidium bromide," Nucl. Acids. Res. 5:2629-2633 (1978)
JR		Larock, "Organomercurials in Organic Synthesis," Tetrahedron 38:1713-1754 (1982)

EXAMINER	DATE CONSIDERED
/Jezia Riley/	08/17/2006

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE
JR		6 3 3 8 9 5 4		Gemen, B.			
JR		5 6 4 6 2 6 4		Glazer, et al			
JR		5 2 4 8 6 1 8		Haces, A.			
JR		5 7 3 0 8 4 9		Hamby, et al			
JR		4 7 0 7 4 5 4		Hendrix, JL			
JR		5 4 6 4 7 4 1		Hendrix, JL			
JR		5 9 9 4 0 5 6		Higuchi, RG			
JR		5 0 4 7 5 1 9		Hobbs, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANSLATION YES	TRANSLATION NO
JR	EP	1 2 7 5 7 3 7	1/15/03	Rabbani, et al	A1			
JR	EP	1 3 4 4 8 3 5	11/17/03	Rabbani, et al	A1			

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

JR		Lee, L.G., et al., "DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments," Nucl. Acids Res. 20:2471-2488 (1992)
JR		Liu, H., et al., "PCR amplification using deoxyinosine to replace an entire codon and at ambiguous positions," Biotechniques 16:24-26 (1994)
JR		Liu, D., et al., "Stable human immunodeficiency virus type 1 (HIV-1) resistance in transformed CD4+ monocytic cells treated with multitargeting HIV-1 antisense sequences incorporated into U1 snRNA," J. Virol 71:4079-4085 (1997)
JR		Loakes, D., et al., "5-Nitroindole as an universal base analogue," Nucl. Acids Res. 22:4039-4043 (1994)
JR		Loakes, D., "The applications of universal DNA base analogues," Nucl. Acids Res. 29:2437-2447 (2001)

EXAMINER	/Jezia Riley/	DATE CONSIDERED	08/17/2006

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO-PRIATE
JR		6228578		Impraim, et al			
JR		5554516		Kacien, et al			
JR		5948648		Kahn, et al			
JR		5945483		Kwok, et al			
JR		5945526		Lee, et al			
JR		5118801		Lizardi, et al			
JR		5130238		Malek, et al			
JR		4683202		Mullis, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANS-LATION YES	TRANS-LATION NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

JR		Maulding, D.R., et al., "Chemiluminescence from Reactions of Electrophilic Oxamides with Hydrogen Peroxide and Fluorescent Compounds," J. Org. Chem.33:250-254 (1968)
JR		Moan, J., et al., "Porphyrin photosensitization and phototherapy," Photochem. Photobio. 43:681-690 (1986)
JR		Mujumdar, R.B., et al., "Cyanine dye labeling reagents containing isothiocyanate groups," Cytometry 10:11-19 (1989)
JR		Mujumdar, R.B., et al., "Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters," Bioconjugate Chemistry 4:105-111 (1993)
JR		Nichols, et al., "A universal nucleoside for use at ambiguous sites in DNA primers," Nature 369:492-493 (1994)
JR		Okayama, H., et al., "High efficiency cloning of full length cDNA," Mol. Cell. Biol. 2:161 (1982)

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO-PRIATE
JR		5 8 6 6 3 3 6		Nazarenko, et al			
JR		6 1 1 4 3 5 0		Randall, et al			
JR		6 1 1 0 6 3 0		Reddy, et al			
JR		6 0 0 1 5 7 3		Roalent, C.			
JR		5 7 0 7 5 5 9		Schaap, et al			
JR		6 3 2 3 3 3 7		Singer, et al			

FOREIGN PATENT DOCUMENTS

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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

JR		Rieke, R.D., "The preparation of highly reactive metals and the development of novel organometallicreagents," Aldrichimica Acta 33:52-60 (2000)
JR		Robins, M.J., et al., "Nucleic Acid Related Compounds. 39. Efficient Conversion of 5-Iodo to 5-Alkynyl and Derived 5-Substituted Uracil Bases and Nucleosides," J. Org. Chem. 48:1854-1862 (1983)
JR		Schaap, et al., "Chemical and Enzymatic Triggering of 1,2-Dioxetanes. 1:Aryl Esterase-Catalyzed Chemiluminescence from a Naphthyl Acetate-Substituted 1,2-Dioxetane," Tetrahedron Letters 28:935-938 (1987)
JR		Schaap, A.P., et al., "Chemical and Enzymatic Triggering of 1,2-Dioxetanes. 3: Alkaline Phosphatase-Catalyzed Chemiluminescence from an Aryl Phosphate-Substituted Dioxetane," Tetrahedron Letters 28:1159-1163 (1987)
JR		Selinger, D.W., et al., "RNA expression analysis using a 30 base pair resolution *Excherichia coli* genome array," Nature Biotech. 18:1262-1268 (2000)
JR		Shibahara, S., et al., "Site-directed cleavage of RNA," Nucl. Acids Res. 15:4403-4415 (1987)

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EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO-PRIATE
JR		4 8 6 8 1 0 3		Stavrianopolous, et al			
JR		4 9 5 2 6 8 5		Stavrianopolous, et al			
JR		4 9 9 4 3 7 3		Stavrianopolous, et al			
JR		5 0 1 3 8 3 1		Stavrianopolous, et al			
JR		5 5 7 8 8 3 2		Trulson, et al			
JR		5 1 3 2 2 0 4		Urdea, et al			
JR		5 8 9 1 6 3 6		Van Gelder, et al			
JR		5 2 6 8 4 8 6		Waggoner, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANS-LATION YES	TRANS-LATION NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

JR		Southwick, P.L., et al., "Cyanine dye labeling reagents – carboxymethylindocyanine succinimidyl esters," Cytometry 11:418-430 (1990)
JR		Talaat, A.M., et al., "Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis," Nature Biotech. 18:679-682 (2000)
		~~Tao, et al., "Genomics: Expression Analysis of Escherichia coli Growing on Minimal and Rich Media," J. Bact. 181:6425-6490 (1999)~~
JR		Wieringa, J.H., et al., "Adamantylideneadamantane Peroxide. A Stable 1,2 Dioxetane," Tetrahedron Letters 2:169-172 (1972)
JR		Zhu, Z., et al., "Directly labeled DNA probes using fluorescent nucleotides with different length linkers," Nucl. Acids. Res. 22:3418-3422 (1994)

EXAMINER	/Jezia Riley/	DATE CONSIDERED	08/17/2006

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Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (use several sheets if necessary)	Atty. Docket No. ENZ-61(D1)	Serial No. 10/761,906
	Applicants: Ilan, et al	
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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO-PRIATE
JR		5627027		Waggoner, et al			
JR		6008373		Waggoner, et al			
JR		5270184		Walker, et al			
JR		5455166		Walker, et al			
JR		4711955		Ward, et al			
JR		5455175		Wittwer, et al			
JR		6174670		Wittwer, et al			

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	TRANS-LATION YES	TRANS-LATION NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

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Notice of References Cited	Application/Control No. 10/761,906	Applicant(s)/Patent Under Reexamination STAVRIANOPOULOS ET AL.	
	Examiner Jezia Riley	Art Unit 1637	Page 1 of 1

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*	A	US-6,403,807	06-2002	Singh et al.	548/455
	B	US-			
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	D	US-			
	E	US-			
	F	US-			
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Direct Covalent Mercuration of Nucleotides and Polynucleotides†

R. M. K. Dale,‡ E. Martin,§ D. C. Livingston,§ and D. C. Ward

ABSTRACT: Nucleotides of cytosine and uracil are readily mercurated by heating at 37–50° in buffered aqueous solutions (pH 5.0–8.0) containing mercuric acetate. Proton magnetic resonance, elemental, electrophoretic, and chromatographic analyses have shown the products to be 5-mercuricytosine and 5-mercuriuracil derivatives, where the mercury atom is covalently bonded. Polynucleotides can be mercurated under similar conditions. Cytosine and uracil bases are modified in RNA while only cytosine residues in DNA are substituted. There is little, if any, reaction with adenine, thymine, or guanine bases. The rate of polymer mercuration is, unlike that of mononucleotides, markedly influenced by the ionic strength of the reaction mixture: the lower the ionic strength the faster the reaction rate. Pyrimidine residues in single- and double-stranded polymers react at essentially the same rate. Although most polynucleotides can be extensively mercurated at pH 7.0 in sodium or Tris-acetate buffers, tRNA undergoes only limited substitution in Tris buffers. The mild reaction conditions give minimal single-strand breakage and, unlike direct iodination procedures, do not produce pyrimidine hydrates. Mercurated polynucleotides can be exploited in a variety of ways, particularly by crystallographic and electron microscopic techniques, as tools for studying polynucleotide structure.

The formation of coordination complexes between mercuric ions and nucleotides or polynucleotides has been known for over 20 years (Katz, 1952, 1963; Thomas, 1954; Yamane and Davidson, 1961; Simpson, 1964; Nandi et al., 1965; Gruenwedel and Davidson, 1967; Mansy et al., 1974). These complexes are quasistable, readily reversed by the addition of agents that act as ligands for Hg^{2+}, such as Cl^- and CN^-, and involve both valences of the Hg^{2+} ion. Although the structure of the complexes were never fully elucidated, Hg^{2+} binding appeared to result from interaction with amino groups and ring nitrogens of the bases (Katz, 1963; Yamane and Davidson, 1961), preferential binding occurring with A-T base pairs (Nandi et al., 1965). Davidson and associates (Nandi et al., 1965; Wang et al., 1965) utilized this selective binding to induce large buoyant density differences in Cs_2SO_4 between DNAs with different base compositions, or between single- and double-stranded DNA. Covalent[1] mercuripolynucleotides could have even greater utility in the selective separation of polymers, in the structural analysis of polynucleotides or polynucleotide-protein complexes, or in electron microscopic methods of gene mapping, provided the mercury substituents are (1) reasonably stable and (2) do not significantly distort the polymer structure. The high electron and buoyant density of the mercury atom and its affinity for free sulfhydryl groups (on proteins or chromatographic supports) would confer upon modified polymers unique physical properties which can be exploited.

We recently reported the preparation of covalent mercurinucleotides of cytosine, uracil, and 7-deazadenine (Dale et al., 1973). The nucleoside 5-triphosphates of these compounds were, in the presence of appropriate mercaptans, excellent substrates for numerous nucleic acid polymerases. Although covalently mercurated polynucleotides can be prepared enzymatically, the mild reaction conditions used for synthesizing the modified nucleotides suggested that direct polymer mercuration could be achieved as well. In this and the accompanying paper (Dale and Ward, 1975) we (1) detail the synthesis, structural characterization, and properties of the 5-mercuripyrimidine compounds, (2) describe methods for controlled direct mercuration of DNA and RNA polymers, (3) report some of the physical and biological properties of both enzymatically and directly mercurated polynucleotides, and (4) describe a method for the selective and quantitative fractionation of polynucleotide sequences complementary to any mercurated polymer probe by rapid chromatography of mercurated hybrids on columns of sulfhydryl-Sepharose. The studies reported here demonstrate that the bulky and potentially reactive mercury atoms do not significantly alter the structure of the polynucleotide (the mercury atom is located in the major groove of polymer duplexes) nor do they interfere with the ability of the polymers to interact with polymerases, nucleases, and other polynucleotide binding proteins. Mercuripolynucleotides, therefore, appear to be suitable probes for a variety of structural studies.

Materials and Methods

Nonradioactive nucleosides and nucleotides were purchased from Sigma and P.L. Laboratories. Radiolabeled nucleotides (^3H and ^{32}P) and [^{203}Hg]mercuric acetate were obtained from New England Nuclear Corporation. Poly(U), poly(C), poly(A), poly(G), dinucleoside monophosphates, and calf thymus DNA (Type V) were products of Sigma. *Escherichia coli* and yeast bulk tRNAs were obtained from Schwarz/Mann. Purified yeast phenylalanyl-tRNA was kindly provided by Dr. Alex Rich. Double-stranded RNAs (Reo type 3 (dearing) and the replicative

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[1] Although metal coordination complexes contain "coordinate-covalent" bonds, in this and the following paper the term covalent is used to describe only carbon-bound mercury atoms.

form of Qβ bacteriophage) were the gifts of Dr. Aaron Shatkin and Dr. Dan Kolakofsky, respectively. rRNA was prepared from *E. coli* MRE 600 ribosomes (gift of Margot Schaechtman) and the 23S and 16S parts were separated by velocity sedimentation in sucrose gradients. T_7 was grown on *E. coli* SY106 and purified by CsCl density centrifugation and the DNA isolated by phenol extraction. Purified f1 DNA and R17 RNA were the generous gifts of Richard Anderson and Dr. Joan Steitz, respectively. Mercuric salts, chromatographic resins, and other reagents were obtained from regular commercial sources.

Proton magnetic resonance spectra were recorded on a Joel M-100 spectrometer. We thank David Kabakoff for assistance with these measurements. Chemical shifts were measured relative to an external tetramethylsilane standard.

Ultraviolet spectra were recorded on a Cary 15 spectrophotometer. Routine spectral analyses were done using a Beckman 25K recording spectrophotometer.

Thin-layer electrophoresis was performed on a Brinkman-Desaga TLC apparatus using Eastman-Kodak cellulose plates (13255) without fluorescent indicator. All ascending thin-layer chromatography separations were done on the same type cellulose support. Elemental analyses were performed by Baron Consulting Corporation, Orange, Conn.

Sulfhydryl-Sepharose 6B was prepared according to the procedure of Cuatrecasas (Cuatrecasas, 1970). The resin contained 8.1 μmol of sulfhydryl groups/ml, as determined by titration with 5,5'-dithiobis[2-nitrobenzoic acid] (Ellman, 1959). Thiol/CPG-550, a controlled pore glass bead resin containing 30 μmol of sulfhydryl groups/ml, was purchased from Pierce Chemical Company.

Since the rate and extent of polynucleotide mercuration are, unlike that of the mononucleotides, extremely dependent on the ionic strength of the reaction mixture (see text), polymer mercuration conditions will depend on the level of mercuration desired and on the length of time one wishes to expose the polymer to elevated temperatures. The following procedure, however, has been employed as our general method for quantitative mercuration of pyrimidine nucleotides, although other conditions can be used with equally satisfactory results. The compound to be mercurated is dissolved in sodium acetate buffer (pH 6.0, 0.1 *M*) at a concentration of 0.02 *M*. An equal volume of 0.10 *M* mercuric acetate (dissolved in the same pH 6.0 acetate buffer) is added to the nucleotide solution and the mixture heated at 50° for 3 hr. After cooling, the mercurinucleotides are chromatographed on columns of DEAE-cellulose (bicarbonate) using a linear gradient of triethylammonium bicarbonate as the eluent. The mono-, di-, and triphosphates of Hg-C[2] and Hg-U elute at approximately 0.1, 0.22, and 0.35 *M* salt, respectively. The mercurinucleotide fractions are pooled and desalted by rotary evaporation. After washing several times with methanol, the product is dissolved in water, adjusted to pH 7.0 with dilute ammonium bicarbonate, and stored at −20°. To prepare samples for long term storage or for elemental analysis, the nucleotides were chromatographed on columns of DEAE-cellulose (chloride), eluting with a 0–0.4 *M* gradient of lithium chloride. Fractions containing nucleotide were concentrated by rotary evaporation, and the nucleotide was precipitated by the addition of four volumes of acetone. The precipitates were collected by filtration, washed twice with ethanol–ether (1:4) and twice with ether, then dried in vacuo over sodium hydroxide pellets.

Mercurated pyrimidine nucleosides are only limitedly soluble in aqueous solutions (less than 1–3 mg/ml), unlike the parent compounds, and often precipitate during the course of the reaction. To purify, the reaction mixtures are concentrated 3–6-fold by rotary evaporation, the mercurinucleoside is collected by filtration, washed twice with cold 0.1 *M* NaCl, twice with ethanol, and ether, then dried in vacuo.

Unreacted mercuric ions can be removed by passing the reaction mixture through a small column of Chelex 100 resin (Bio-Rad Laboratories) which has been previously washed with 0.1 *M* sodium acetate buffer (pH 6.0) until pH equilibration has been obtained. Chelex 100 has an extremely high affinity for Hg^{2+} ions, binding approximately 0.7 mequiv/ml of resin, but it does not normally adsorb mercurated nucleotides (R-Hg^+). Since Chelex 100 can catalyze a slow demercuration, it is advisable to test the resin before using it in a routine manner and to keep the resin exposure time to a minimum (15–30 min). Chromatography of reaction mixtures on columns of Sephadex G-10 will remove most unreacted Hg^{2+} ions under conditions where no product demercuration occurs. Final purification is then achieved by DEAE-cellulose chromatography.

An alternative procedure for purification, or for measuring product purity, is to chromatograph the reaction mixtures (after removal of free Hg^{2+} ions) on columns of sulfhydryl-Sepharose or sulfhydryl-glass beads. Mercurinucleotides are quantitatively retained on these resins whereas nonmercurated nucleotides are eluted. After washing the resin with 0.1 *M* NaCl to remove any nonmercurated material, the mercurinucleotides are batch eluted with 0.10 *M* sodium cyanide or 0.1 *M* mercaptoethanol. The nucleotide product is precipitated immediately upon elution with four volumes of ethanol or acetone and dried as above. Prolonged exposure to high concentrations of cyanide or mercaptans should be avoided as they induce reductive demercuration when present in large (50–1000-fold) molar excess over the mercurinucleotide.

Radioactive mercurinucleotides are readily prepared by the above procedures using [^{203}Hg]mercuric acetate. Mercury-203 is a relatively inexpensive isotope ($1.20 per mCi in 100mCi lots) which emits β and γ radiation of 0.212 and 0.28 MeV, respectively, with a half-life of 46.6 days. Currently available specific activities (4 Ci/mmol) yield nucleotides that give 10^7 cpm/μg in either β or γ counters; however, with isotopic enrichment specific activities of over 10^9 cpm/μg are possible. Mercurinucleotides radiolabeled with ^{203}Hg are suitable for autoradiography (Figure 5) and for nucleotide binding studies.

Elemental analysis of the mercurated products revealed that in each case only one mercury atom per base was introduced. Characterization of the compounds (see "Results") has shown the mercury substituent to be on the 5 position of the pyrimidine ring. Typical analytical results are given below for Hg-U and Hg-UMP, respectively. Calcd for $C_9N_2O_6H_{11}HgCl$: C, 22.54; N, 5.84; O, 20.04; H, 2.30; Hg, 41.87; and Cl, 7.41. Found: C, 22.16; H, 2.40, N, 5.56; Hg, 41.30. Calcd for $C_9N_2O_9H_{10}HgPClNa_2 \cdot H_2O$: C 17.41; N, 4.51; H, 1.93; Hg 32.34. Found: C, 17.31; N, 4.70; H, 2.15; Hg, 32.48.

[2] Abbreviations used are: Hg-U, Hg-C, Hg-UMP, Hg-CMP, etc., the 5-mercuri derivatives of U, C, UMP, CMP, etc., as the chloride or acetate salts.

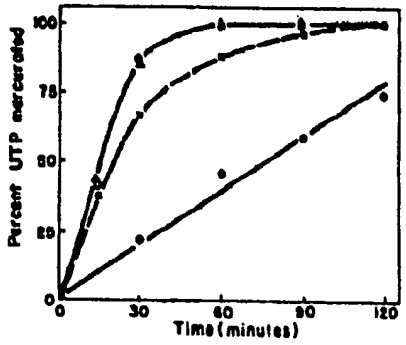

FIGURE 1: Kinetics of UTP mercuration in 0.1 *M* sodium acetate buffer: pH 4.0 (●), pH 5.0 (■), pH 6.0 (○), and pH 7.0 (▲). Each reaction (10 ml), run in duplicate, contained 6×10^{-4} *M* UTP and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (specific activity, 1.7×10^{6} cpm/μmol); 1.0-ml aliquots were removed at the indicated times and processed as described under Materials and Methods.

To follow the kinetics of nucleotide mercuration reactions the following protocol was used. Duplicate 10-ml reactions were incubated in the desired buffer containing 5×10^{-4}–5×10^{-3} *N* nucleotide and a fivefold molar excess of [^{203}Hg]mercuric acetate ($1\text{-}5 \times 10^{6}$ cpm/μmol). At appropriate times, 1.0-ml aliquots were removed and added to a 1.0-ml suspension of Chelex 100 resin on ice. The resin, prior to use, was adjusted to pH 7.0 by extensive washing with 0.1 *M* sodium acetate buffer (pH 7.0), and tested to be sure it did not catalyze demercuration. The nucleotide-Chelex suspension was stirred or shaken for 5 min, then the resin allowed to settle. The supernatant liquid was removed and treated twice more with Chelex as described above. After the third treatment the resin was removed by filtration, the nucleotide concentration was determined spectrophotometrically, and the ^{203}Hg label not adsorbed to Chelex determined by counting 0.1-ml samples in 3.0 ml of Aquasol using either a Packard scintillation counter or an Intertechnique CG30 automatic γ-counter. Control reactions containing only [^{203}Hg]mercuric acetate were processed in an identical manner to provide the background level of unadsorbed isotope. In all cases greater than 99% of the added [^{203}Hg]mercuric acetate was adsorbed by the Chelex treatment. After subtraction of the background counts the ^{203}Hg cpm/OD_{260} ratio was used to calculate the percentage mercuration. The results of duplicate reactions agreed to within ±2.5%. The covalent nature of the mercuration product was confirmed by electrophoresis at pH 7.5 in the presence and absence of added mercaptoethanol (see text).

Results

Covalent Mercuration of Mononucleotides. Although both purine and pyrimidine nucleotides rapidly form quasi-stable mercury–nucleotide complexes at neutral pH (Yamane and Davidson, 1961; Katz, 1963), only pyrimidine nucleotides undergo facile covalent mercuration. Uracil and cytosine derivatives are quantitatively mercurated within 90 min when heated at 50° in 0.1 *M* sodium acetate buffer (pH's 5.0, 6.0, or 7.0) containing a five- to sixfold molar excess of mercuric acetate. The kinetics of UTP mercuration (Figure 1) are characteristic of all U and C nucleotides. In contrast, less than 2% of thymine, 1% of adenine, and 3% of guanine containing nucleotides are modified after a 24-hr reaction under identical conditions (Table I). Since the purine and thymine nucleotides were not exhaustively purified by chromatographic or electrophoretic means before or after mercuration, the values given represent an upper limit of substitution and may reflect, in part, the purity of the starting material. It is apparent, however, from these studies that nucleotide derivatives of uracil and cytosine react at least 100–200 times faster than other nucleotides. It is interesting to note that pyrimidines with a substituent on the C-5 ring position (thymidine and pseudouridine) are as inactive as purines toward mercuration. The pyrimidine specificity of the mercuration reaction is similar to that seen with thalic chloride catalyzed iodination (Commerford, 1971; Prensky et al., 1973, Scherberg and Refetoff, 1974). However, unlike direct iodination, mercuration proceeds with equal facility on both U and C bases. In addition, whereas extensive uracil hydrate formation occurs in the iodination reaction (Commerford, 1971; Scherberg and Refetoff, 1974) no hydration of the 5-6 double bond occurs during mercuration. Since mercurinucleotides can be rapidly converted to iodonucleotides in high yields (see below), iodination via mercuri intermediates may offer some advantages in the preparation of iodinated nucleotides and polynucleotides.

Table I: Specificity of the Mononucleotide Mercuration Reaction.[a]

Substrate	Percent Mercurated Product: 2-hr Reaction	24-hr Reaction
UTP	100	100
CTP	100	100
ATP	0.45	0.91
GTP	0.58	3.0
TTP	1.7	1.8
Pseudouridine MP		1.3

[a] Reactions (2.0 ml) contained 6×10^{-4} *M* substrate and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (specific activity, 1.7×10^{6} cpm/μmol) in 0.1 *M* sodium acetate buffer (pH 6.0). After heating for the indicated time at 50° the mixtures were cooled on ice, diluted to 10 ml with water, applied to 1×3 cm columns of DEAE-cellulose (bicarbonate form), and washed to remove most unreacted mercuric acetate. The nucleotides were batch eluted with 1.0 *M* triethylammonium bicarbonate, desalted by rotary evaporation, and dissolved in 1.0 ml of water. The nucleotide content of each sample was determined spectrophotometrically and a known quantity of the nucleotide electrophoresed at pH 7.5 with and without added mercaptoethanol. The percent mercuration values were calculated from the ^{203}Hg-radiolabel associated with uv absorbing material after mercaptan treatment. Similar values were obtained by the Chelex 100 adsorption techniques described under Materials and Methods.

Although purine bases react extremely slowly at neutral pH, they can be mercurated in low yield (10–20%) by refluxing in 50% acetic acid for extended periods (18–24 hr). These extreme reaction conditions, however, preclude direct mercuration of purine compounds with labile pyrophosphate or phosphodiester linkages. Although the normal purine nucleotides are poor substrates for mercuration, the 7-deazapurine analogs of A and G are mercurated as readily as the pyrimidines (Dale et al., 1973).

Mercuric acetate was selected as the mercurating agent because it is (1) highly reactive (Makarova and Nesmeyanov, 1967), (2) highly soluble in aqueous solutions, (3) readily available, and (4) inexpensive. Other mercuric salts are, however, suitable agents. These include mercuric nitrate, mercuric perchlorate, mercuriacetamide, and mercur-

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Table II: Effect of Temperature, Buffer Concentration, and pH on the Mercuration of UTP.[a]

Buffer	Buffer Concn (M)	Temp (°C)	Reaction Time (min)	UTP Covalently Mercurated (%)
Sodium acetate, pH 6.0	0.10	37	15	20
pH 6.0	0.10	50	15	38
pH 6.0	0.10	60	15	72
pH 6.0	0.005	50	90	100
pH 6.0	0.05	50	90	98
pH 6.0	0.50	50	90	97
Sodium acetate, pH 4.0	0.1	50	30	26
pH 5.0	0.1	50	30	67
pH 6.0	0.1	50	30	84
pH 7.0	0.1	50	30	86
Tris-acetate, pH 6.0	0.1	50	30	7.5
pH 7.0	0.1	50	30	10.7
pH 8.0	0.1	50	30	9.2

[a] The UTP and [^{203}Hg] mercuric acetate concentrations were the same as those given in the legend to Figure 1. The reactions were terminated and processed as described under Materials and Methods.

itrinitromethane, all of which function with an efficiency similar to that of mercuric acetate. Mercuric oxide and mercuric sulfate are poor reagents since they are only slightly water soluble. Mercuric halide salts (e.g., $HgCl_2$, $HgBr_2$) and mercuric cyanide are essentially inactive; no appreciable level of covalent mercuration could be seen in our test systems even after extensive reaction times.

To minimize the hydrolytic degradation of potential substrates we have chosen reaction conditions which afford high yields when run at or near neutral pH and at relatively low temperatures (50° or less). The rate of mercuration can, however, be increased by utilizing higher temperatures or by increasing the mercuric acetate/nucleotide ratio. As shown in Table II, the mercuration of UTP, which is typical of both U and C monomers, exhibits a temperature coefficient of approximately 1.9 and a pH optimum near pH 7.0.

Mercuration reactions are done in buffered aqueous solutions to prevent the mixtures from becoming acidic as the reaction ($R\text{-}H + HgX_2 \rightarrow R\text{-}HgX + HX$) proceeds. Sodium acetate, sodium citrate, potassium citrate-phosphate, and borate-sodium hydroxide buffers have been found satisfactory. Buffers containing amine salts or halide ions significantly reduce or totally inhibit the reaction. For example, mercurations done in Tris-acetate buffer (pH 6.0) proceed at one-tenth the rate of reactions carried out in an equivalent concentration of sodium acetate (pH 6.0) buffer (Table II). Tris-chloride, glycine-acetate, and glycine-NaOH buffers almost totally inhibit mercuration. Although the buffer concentration can be varied considerably (0.0005–0.50 *M*) without significantly affecting mononucleotide mercuration (Table II), changes in the ionic strength of the reaction profoundly influence both the rate and extent of polynucleotide mercuration (see below). The use of buffers of low ionic strength (<0.02 *M*) decreases the concentrations of both reactants that can be effectively employed, since addition of mercuric salts to concentrated solutions of nucleotide (particularly polynucleotide) in dilute buffer causes an almost immediate precipitation of noncovalent mercurinucleotide salts. Once precipitated the rate of covalent mercuration is significantly reduced. Precipitation problems, occasionally seen with oligo- and polynucleotides containing a high C and/or G content, can be circumvented by increasing the ionic strength or the pH of the reaction buffer.

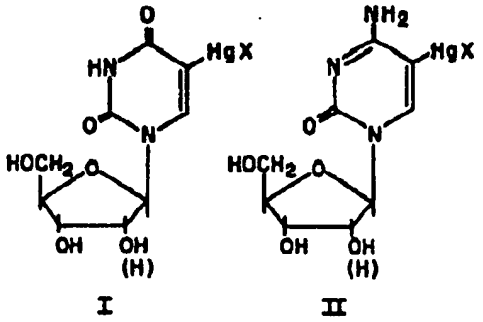

FIGURE 2: Structure of 5-mercuriuridine (deoxyuridine), I, and 5-mercuricytidine (deoxycytidine), II. The mercury ligand, X, may be Cl^-, CN^-, $R\text{-}S^-$, or other appropriate counterion.

Structural Characterization of Mercurinucleotides. The mono-, di-, and triphosphates of U, C, dU, and dC were converted to the corresponding mercuri derivative as described under Materials and Methods. Although elemental analyses of the mercurinucleotides indicated that each contained a single mercury atom, the site of mercuration had to be established. Since acetoxymercuration reactions proceed via electrophilic substitution (Makarova and Nesmeyanov, 1967), the likely position of attachment is on the C-5 carbon, the most electronegative carbon of the pyrimidine nucleus (Pullman and Pullman, 1969). Three independent methods were used to establish that the products of mercuration are indeed 5-mercurinucleotides (Figure 2).

1. PROTON MAGNETIC RESONANCE (PMR): The mercurinucleotides as originally isolated (see Materials and Methods) contain either bicarbonate or chloride as the counterion to the bound mercury. When solutions of such nucleotide (0.05–0.1 *M* in D_2O) were analyzed by PMR no resonance spectrum could be detected, even at the highest sensitivity settings. In contrast, the corresponding nonmercurated parent compounds gave excellent spectra with the expected chemical shifts. In order to obtain an appreciable PMR signal from the mercurinucleotides, an equivalent molar concentration of a mercaptan, mercaptoethanol, had to be added to the D_2O solution. Since Hg^{2+} and $R\text{-}Hg^+$ ions can form quasistable complexes with phosphate and aromatic amines, we believe that, in the absence of mercaptan and at high nucleotide concentration, intermolecular interactions generate polymeric charge complexes $[PO_4^{-}\text{-}R\text{-}Hg^{+}\text{-}PO_4^{-}\text{-}R\text{-}Hg^{+}]n$ or $[R\text{-}Hg\text{—}N\text{-}Ar\text{-}Hg\text{—}N\text{-}Ar]_n$ which abolishes the monomer resonance. Addition of mercaptoethanol disrupts the polymeric complex by forming the more stable mercurithioethanol nucleotides. The complex forming ability of mercurinucleotides can also be demonstrated spectrally (see below).

Figure 3 shows the downfield PMR spectra of UMP before and after mercuration. Only the chemical shifts assigned (Jardetzky and Jardetzky, 1960; Schweizer et al., 1968) to the H-5 (6.37 ppm) and H-6 (8.40 ppm) of the pyrimidine ring and the H-C_1 (6.41 ppm) of the sugar are illustrated. After mercuration the H-5 doublet has disappeared and the H-6 doublet collapsed to a singlet. Similar changes in the chemical shifts of the H-5 and H-6 protons of cytidine compounds were seen on mercuration. Since the resonances of the other sugar protons were not altered we

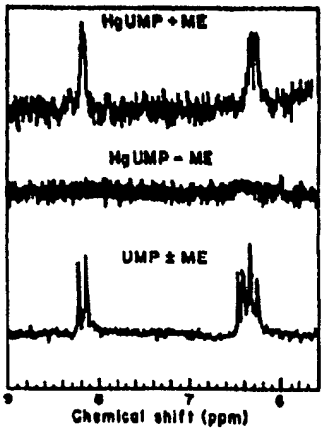

FIGURE 3: Downfield PMR spectra of UMP and Hg-UMP in the presence and absence of 2-mercaptoethanol (ME). Nucleotide concentration was 0.1 *M* in D_2O; mercaptoethanol, where present, 0.1 *M*. See text for discussion.

conclude that the mercury atom is attached to the C-5 ring carbon.

2. MERCURATION OF TRITIATED NUCLEOTIDES. When [^{3}H]CMP and [^{3}H]UMP (tritiated specifically in the C-5 position) are treated with [^{203}Hg]mercuric acetate under the standard reaction conditions, the ^{3}H-radiolabel is quantitatively lost while 1 equiv of ^{203}Hg-radiolabel is acquired. In contrast, no tritium label is removed upon mercuration of [6-^{3}H]uridine. The loss of the C-5 tritium upon mercuration confirms the PMR results and suggests that the reaction proceeds via the classical electrophilic substitution mechanism ($RH + HgX_2 \rightarrow RHgX + HX$).

3. CONVERSION OF HG-UMP AND HG-CMP TO 5-IODO-UMP AND 5-IODO-CMP. Many organomercurials are known to be susceptible to demercuration by a variety of electrophilic reagents, including halogens (Jensen and Rickborn, 1968). Reaction of mercurinucleotides with elemental iodine might therefore be expected to yield the corresponding 5-iodonucleotides. Hg-UMP and Hg-CMP were dissolved in water or 0.05 *M* KI (to enhance the solubility of I_2 in water) and treated with a 50% aqueous-alcoholic solution of I_2. After standing at room temperature for 1 hr the reaction mixtures were extracted three times with chloroform, the residual aqueous solutions filtered through Whatman No. 1 paper, and the filtrates chromatographed on columns of DEAE-cellulose. The reaction products were eluted with triethylammonium bicarbonate (0.12 *M*) and subjected to spectral and chromatographic analyses against authentic samples of 5-iodo-UMP and 5-iodo-CMP. Demercuration of Hg-UMP and Hg-CMP by I_2 gave, in near quantitative yields, nucleotides which were identical in all respects with the corresponding iodinated reference compounds. The demercuration reaction is catalyzed by a variety of electrophiles and we have prepared a number of halogenated and tritiated nucleotide compounds by this general method, the details of which will be published elsewhere.

Properties of Mercurated Nucleotides. Mercurated pyrimidine nucleosides and nucleotides, although normal in many respects, do possess a number of unusual characteristics. For example they form gels when dissolved in water at high concentrations, most likely a consequence of intermolecular interactions of the type described above. Although

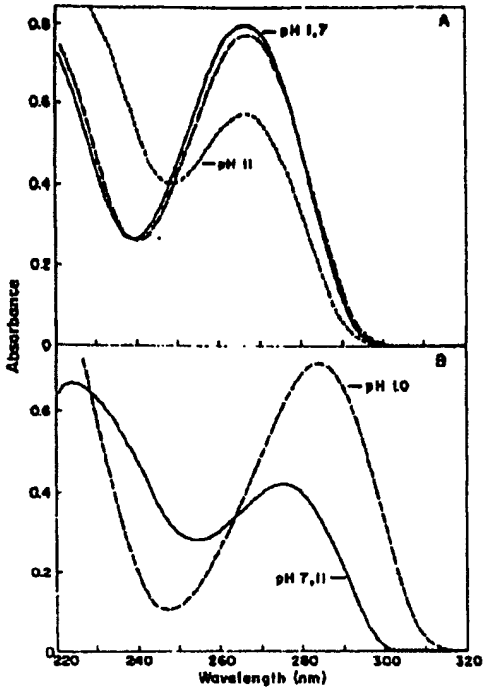

FIGURE 4: The uv spectra of Hg-UMP (A) and Hg-CMP (B) in 1.0 *M* NaCl containing either (a) 0.01 *M* Tris-HCl buffer (pH 7.0), (b) 0.1 *M* HCl, or (c) 0.001 *M* NaOH. (- - -) Spectrum of Hg-UMP in 0.01 *M* Tris-HCl, (pH 7.0) without added sodium chloride. The molar extinction coefficients of Hg-UMP and Hg-CMP at pH 7.0 (in high NaCl) are 10,100 and 9200, respectively.

the viscosity drops on dilution, complex formation still occurs at 10^{-4}-10^{-5} *M* as judged by spectrophotometric measurements. The observed uv absorption of mercurated nucleotides is, unlike that of the parent compounds, salt and temperature dependent. Addition of sodium chloride to a 5×10^{-5} *M* solution of Hg-UMP (in water) increases the overall absorption by 5-15% and induces a very slight blue shift in the long wavelength region of the spectrum. To obtain the maximum increase in absorption a final NaCl concentration of approximately 0.5-1.0 *M* is required. However, strong mercury ligands, such as mercaptoethanol or cyanide (Simpson, 1961), require only a 2-3-fold molar excess. Elevated temperatures (>60°) also give similar small absorption increases which, in contrast to the salt effect, are reversible. The spectral changes resemble those observed on denaturation of polynucleotides. The aggregation which occurs in the absence of an appropriate counterion for the remaining mercuric ionization should, therefore, be taken into consideration when preparing or studying mercurinucleotide solutions.

The spectra of Hg-CMP and Hg-UMP in 1 *M* NaCl (Figure 4) are typical of all mercurated C and U compounds. The absorption maxima occur at longer wavelengths (by 5 nm) than those of the parent pyrimidine nucleotides, although the molar extinction coefficients are essentially identical. The 5-nm spectral difference is independent of the pH at which the spectrum is determined. Although the entire spectrum of Hg-U compounds appears to be red shifted, the Hg-C compounds also lack the broad spectral shoulder in the 230-240-nm region characteristic

Table III: Electrophoretic Properties of Mercurated Nucleotides.[a]

Nucleotide[b]	Electrophoretic Mobility Relative to the Corresponding Nonmercurated Parent Compound			
	pH 3.5		pH 7.5	
	No ME	+ ME	No ME	+ ME
Hg-UMP	0.89	0.65	0.55	0.90
Hg-UDP	0.88	0.84	0.67	0.92
Hg-UTP	0.90	0.87	0.65	0.94
Hg-CMP	0–0.10	0.80	0.40	0.88
Hg-CDP	0.80	0.75	0.72	0.92
Hg-CTP	0.94	0.90	0.82	0.95

[a] Electrophoretograms were run on 20 × 20 cm cellulose thin-layer plates for 3 hr at 300 V in either 0.05 *M* sodium citrate buffer (pH 3.5) or in 0.05 *M* ammonium bicarbonate adjusted to pH 7.5 with CO_2. Aliquots of stock mercurinucleotide solutions were removed and treated at room temperature with a tenfold molar excess of mercaptoethanol for 5 min before spotting. Nucleotide spots were localized by uv adsorption or by autoradiography. [b] The electrophoretic properties of mercurideoxynucleotides are identical with the corresponding ribo-compound.

Table IV: Chromatographic Properties of Mercurated Ribonucleotides in 95% Ethanol–Water (70:30).[a]

Compound	R_f Value	Compound	R_f Value
UMP	0.73	CMP	0.70
Hg-UMP	0.37	Hg-CMP	0.16
UDP	0.70	CDP	0.57
Hg-UDP	0.48	Hg-CDP	0.47
UTP	0.66	CTP	0.53
Hg-UTP	0.54	Hg-CTP	0.39

[a] All chromatograms were run in an ascending fashion on cellulose thin-layer plates.

of C derivatives. Addition of strong mercury ligands to salt solutions of mercurated nucleotides do not induce further spectral changes even though they remove the ionic character of the mercury substituent by forming covalent ligand-mercurinucleotides. Although mercuration reactions can be followed spectrally by monitoring the increase in absorption at 290 nm (for U compounds) or 295 nm (for C compounds), caution should be exercised as the noncovalent mercury-nucleotide complexes possess spectra similar to the covalent derivatives. One can distinguish, however, between the two reactions since the noncovalent complexes are disrupted by the addition of CN^- or Cl^- ions (Yamane and Davidson, 1961; Nandi et al., 1965).

Spectrophotometric titrations of Hg-UMP and Hg-CMP demonstrate that the pK_a values of the ionizable ring protons are not significantly altered as a consequence of mercuration. The observered pK_a values were: Hg-CMP, 4.6; CMP, 4.5; Hg-UMP, 9.7; UMP, 9.6. The hydrogen bonding characteristics of mercurated pyrimidine nucleotides should, therefore, be similar to those of the normal pyrimidines. The facility with which mercurated pyrimidine nucleoside 5′-triphosphates are enzymatically polymerized (Dale et al., 1973), and the thermal denaturation profiles of polymer duplexes containing one Hg atom per base pair (Dale and Ward, 1975), support this contention.

Mercurated nucleotides are readily distinguished from the parent compounds on the bases of their chromatographic and electrophoretic properties (Tables III and IV). When electrophoresed at pH 7.5 as the chloride or carbonate salts, the mercurinucleotides exhibit a significantly slower electrophoretic mobility than that of the corresponding nonmercurated nucleotide. Addition of sodium cyanide or mercaptoethanol to the samples prior to electrophoresis, or inclusion of mercaptoethanol in the electrophoresis buffer, increases the mobility of all mercurinucleotides to approximately 85–95% that of their nonmercurated counterparts. Although the mobility of most mercurinucleotides at pH 3.5 is only slightly altered on the addition of mercaptoethanol, the migration of Hg-CMP increases from 0–10 to 80% that of CMP while Hg-UMP migration, surprisingly, decreases significantly. The increase in electrophoretic mobility at pH 7.5 in the presence of a mercaptan (see Table III and Figure 5) is diagnostic of covalent mercurinucleotides. Electrophoresis also provides a convenient method for quantitating the level of Hg^{2+} (or noncovalent mercury–nucleotide complex) contamination in samples containing ^{203}Hg radiolabel. Prolonged exposure of the mercurinucleotides to a large (50–1000-fold) excess of mercaptoethanol or cyanide should, however, be avoided since these ligands can catalyze reductive demercuration. Mercuricytosine derivatives have been found to be considerably more sensitive to this demercuration process than mercuriuracil compounds. Although somewhat labile in the presence of excess reducing agents, mercurinucleotides are quite stable under the conditions of most biochemical or enzymatic assays and tolerate pH extremes and elevated temperatures with little, if any, degradation (Dale et al., 1973).

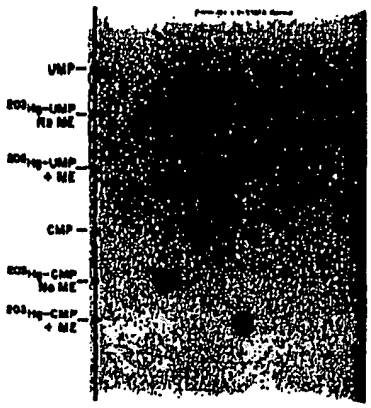

FIGURE 5: Electrophoretic mobility of [^{203}Hg]UMP and Hg-CMP at pH 7.5 in the presence and absence of mercaptoethanol; 0.1 OD_{260} of [^{203}Hg]UMP and [^{203}Hg]CMP (containing 1.1×10^5 and 4.5×10^4 cpm, respectively) were applied to a 20 × 20 cm thin-layer cellulose plate and electrophoresed for 1.5 hr at 300 V. The plate was dried and exposed to Kodak RP/R54 film for 2 hr before development. UMP and CMP markers were localized by uv adsorption.

Direct Mercuration of Polynucleotides. Since the conditions used for mercurating pyrimidine nucleotides were fairly mild, direct polynucleotide mercuration was examined. Poly(U) was used as the test polymer since it possesses little ordered secondary structure at neutral pH, nor does it form self-duplexes at pH 5.0 or below like poly(C) and poly(A) (Michelson et al., 1967). As shown in Figure 6, poly(U) can be quantitatively mercurated by heating for 2 hr at 50° in 0.005 *M* sodium acetate buffer (pH 6.0) containing a sixfold molar excess of mercuric acetate. The rate of poly(Hg-U) formation exhibits a striking and unexpected dependence on the buffer concentration, the rate being significantly greater in low salt buffers (Figures 6 and 7). This reciprocal relationship is in sharp contrast to the

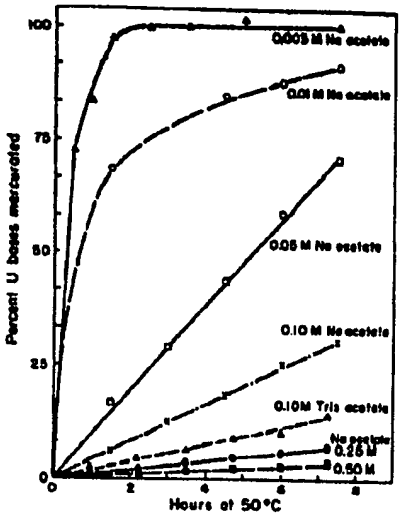

FIGURE 6: The kinetics of poly(U) mercuration at 50° as a function of the buffer concentration. Each 1.0-ml reaction was run in triplicate and, in addition to the indicated concentration of sodium acetate, pH 6.0, or Tris-acetate, pH 6.0, buffer contained 6×10^{-4} *M* poly(U) (dialyzed extensively against water prior to use to remove low molecular weight material) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (specific activity, 1.1×10^{7} cpm/μmol). Control reactions containing [^{203}Hg]mercuric acetate and polymer were run for each buffer concentration and incubated in parallel at 4°. At the indicated times 0.1-ml aliquots were removed and pipetted into 1.0 ml of ice-cold quench buffer (0.01 *M* Tris-HCl (pH 7.5)–0.1 *M* EDTA–1.0 *M* NaCl). The samples were kept on ice for 10 min, then dialyzed at 4° against TNE buffer (0.01 *M* Tris-HCl buffer (pH 7.5)–0.02 *M* NaCl–0.001 *M* EDTA). A maximum of 40 samples were used per 7 l. of TNE buffer. Dialysis was continued for 48 hr with buffer changes every 12 hr, or until the radioactivity in the control reactions gave only background counts. The polymer content of each sample was determined spectrophotometrically; the ^{203}Hg content obtained by counting duplicate 0.05-ml aliquots, drying on GF/A filters, and using a Packard scintillation counter. The percent of bases mercurated was calculated from the ^{203}Hg cpm/$OD_{260\,nm}$ ratio using a molar extinction coefficient of 9800 for poly(U). The results shown are the average of the triplicate reactions; the variability between individual samples was usually less than ±5%.

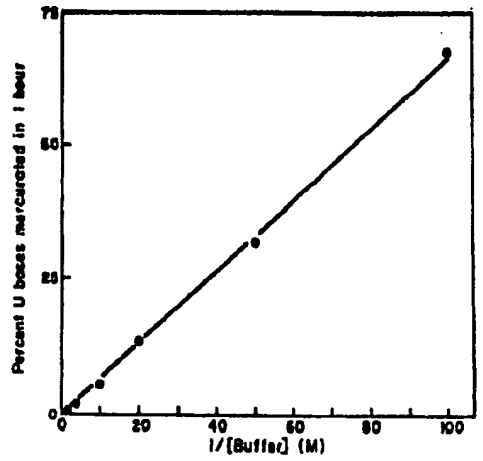

FIGURE 7: The rate of poly(U) mercuration is inversely proportional to the buffer concentrations. Dialyzed poly(U) (6×10^{-4} *M*) and [^{203}Hg]mercuric acetate (1.1×10^{7} cpm/μmol) were reacted for 1 hr at 50° in 0.01, 0.02, 0.05, 0.10, 0.025, and 0.50 *M* sodium acetate buffer (pH 6.0). The reactions were terminated and processed as described in the legend to Figure 6.

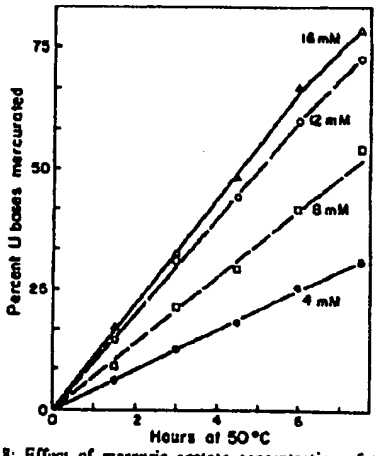

FIGURE 8: Effect of mercuric acetate concentration of the rate of poly(U) mercuration. All reactions (1.0 ml) were run in triplicate in 0.1 *M* sodium acetate buffer (pH 6.0) using 6×10^{-4} *M* dialyzed poly(U) and the indicated concentration of [^{203}Hg]mercuric acetate (9×10^{6} cpm/μmol). Reactions containing the various concentrations of radioactive mercuric acetate but no polymer were run in parallel. Samples were collected and processed as described in the legend to Figure 6.

mercuration of UMP where the rate of reaction is essentially independent of ionic strength (Table II). No convincing arguments can be offered to explain the polynucleotide salt effect. High salt could increase base stacking interactions and thereby alter the electronic character of the uracil base; however, the observation that single- and double-stranded polynucleotides are mercurated at the same rate (see below) seems to make this possibility unlikely. Buffers containing amine salts (e.g., Tris) lower the rate of polymer mercuration but not to the same extent as observed in mononucleotide reactions. Although complete mercuration is obtained within 2 hr under the conditions given in Figure 6, the time of exposure at 50° can be decreased to less than 1 hr by using higher levels of mercuric acetate; the reaction rate increases up to a mercuric acetate/nucleotide ratio of 25–30:1 (Figure 8). Poly(U) mercuration proceeds optimally at neutral pH (pH 6–7) and exhibits a temperature coefficient (Q10) of approximately 2.0–2.2 (Figures 9 and 10). With regard to these parameters, monomer and polymer mercurations are essentially identical. It is apparent from these studies that poly(U) can be extensively modified under conditions where little phosphodiester bond cleavage should occur. Indeed, chromatography of poly(Hg-U) on Sephadex G-200 gave elution profiles which were superimposable on those of the poly(U) starting material. Nucleolytic degradation of poly(Hg-U) and analysis of the resultant nucleotides by chromatography and electrophoresis clearly demonstrate that 5-mercuriuracil bases are the products of mercuration. The characterization and biochemical properties of poly(Hg-U) are presented in the accompanying report (Dale and Ward, 1975).

Polymer mercuration exhibits the same pattern of base specificity as the mononucleotides, as judged by reactions with poly(C), poly(A), poly(G), and poly(T). Since the homopolymers of C, A, and G precipitate in low ionic strength solutions upon the addition of mercuric salts, the reactions were done in 0.1 *M* sodium acetate buffer (pH 6.0) at 50°

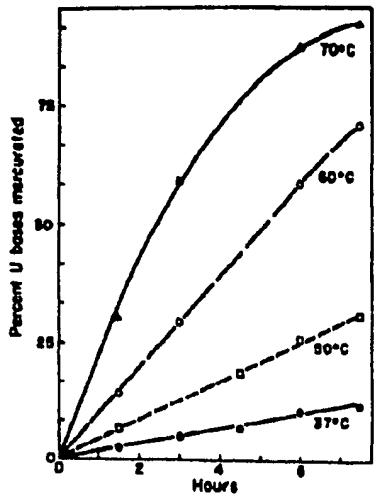

FIGURE 9: Rate of poly(U) mercuration as a function of temperature. All reactions (1.0 ml) were run in triplicate in 0.1 *M* sodium acetate buffer (pH 6.0), with 6×10^{-4} *M* dialyzed poly(U) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (1.2×10^{7} cpm/μmol). Samples were collected and processed, with appropriate 4° controls, as described in the legend to Figure 6.

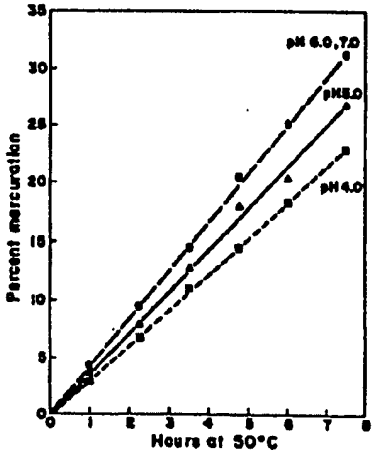

FIGURE 10: Mercuration of poly(U) in 0.1 *M* sodium acetate buffer: pH 4.0 (■), pH 5.0 (▲), pH 6.0 (●), and pH 7.0 (○). Reactant concentrations and work-up are as described in Figure 6.

using only a sixfold molar excess of mercuric acetate. Although these conditions are less than optimal, poly(U) was completely mercurated in 24 hr. In contrast, less than 0.5% of the bases in poly(A), poly(G), and poly(T) were modified (Table V). Poly(C) mercuration proceeded as rapidly as that of poly(U) for several hours. However, the polymer precipitated after 20–40% of the bases had been mercurated. This insolubility problem has prevented our obtaining fully mercurated poly(C) for physical studies. Naturally occurring polynucleotides do not, in general, exhibit the insolubility of the homopolymers and extensive substitution can be achieved in low ionic strength solutions. Figure 11 illustrates the mercuration kinetics of 23S rRNA, bacteriophage R17 RNA, single-stranded fd DNA, and native T_7 DNA. It is apparent from the data that the rate of mercuration with heteropolymers is considerably slower than that observed for poly(U) or poly(C) under comparable conditions. Preliminary studies with dinucleoside monophosphates, UpA, UpG, etc., suggest that there may be a nearest neighbor base effect on the rate of pyrimidine mercuration (R. M. K. Dale and D. C. Ward, unpublished results) which could account, at least in part, for the observed rate difference. It is unlikely that secondary structure plays a dominant role in rate regulation since both native and heat-denatured DNA (T_7 and calf thymus) and RNAs (Reo virus and QβRF) react at identical rates and to the same extent (Table VI). The observation that single- and double-stranded polymers are mercurated with identical kinetics may appear at first somewhat surprising since most direct chemical modifications of polynucleotides, for example, iodination (Commerford, 1971), proceed more rapidly with

Table V: Covalent Mercuration of Homopolymers.[a]

Polymer	Percent Bases Mercurated	
	2 hr	24 hr
Poly(U)	11.3	100
Poly(C)	10.6	ppt[b]
Poly(A)	<0.05	0.27
Poly(G)	<0.05	0.43
Poly(T)	<0.05	0.13

[a] Reactions (1.0 ml) containing 0.1-*M* sodium acetate (pH 6.0), 4×10^{-4} *M* polymer, and 2×10^{-3} *M* [^{203}Hg]mercuric acetate (1.6×10^{7} cpm/μmol) were incubated at 50°. 1.0 ml of ice cold quench buffer (0.01 *M* Tris-HCl (pH 7.5)–0.1 *M* EDTA–1.0 *M* NaCl) was added to each tube at the indicated times to terminate the reaction. The samples were then chromatographed on 1 × 30 cm columns of Sephadex G-25 using the high salt quench buffer as eluent. Fractions containing polymer were pooled and dialyzed extensively (see legend to Figure 6) before calculating the percent base mercuration from the ^{203}Hg cpm/OD (adsorption maximum) ratio, using the following molar extinction coefficients: poly(U), 9800; poly(C), 7200; poly(A), 10,500; poly(G), 9500; and poly(T), 9200. [b] Poly(C) precipitates from the reaction mixture after 20–40% of the bases are mercurated.

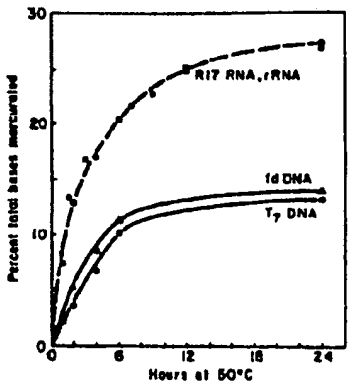

FIGURE 11: Mercuration of R17 RNA (■), 23S rRNA (○), fd DNA (▲), and native T_7 DNA (●). Each 1.0-ml reaction, run in triplicate, contained 0.005 *M* sodium acetate buffer (pH 6.0) and 4×10^{-3} *M* [^{203}Hg]mercuric acetate (1.6×10^{7} cpm/μmol). The polymer nucleotide concentrations were: rRNA and R17 RNA, 4.0×10^{-4} *M*; fd DNA, 4.4×10^{-4} *M*, and T_7 DNA, 6.1×10^{-4} *M*. Concentrations were based on 108 nmol/$OD_{260\,nm}$ for rRNA, R17, and fd DNA and 142 nmol/OD_{260} for native T_7 DNA. Aliquots were removed at the indicated times and processed, with polymer-free controls, as previously described (Figure 6).

Table VI: Single- and Double-Stranded Polynucleotides are Mercurated at the Same Rate.

Polymer	Buffer	Buffer Concn (*M*)	Percent Total Bases Mercurated		
			1 hr	3 hr	5 hr
Native C.T. DNA	Sodium acetate, pH 6.0	0.005	2.88	7.42	10.0
Native C.T. DNA	Sodium acetate, pH 6.0	0.10	1.40	3.49	5.50
Denatured C.T. DNA	Sodium acetate, pH 6.0	0.10	1.55	3.56	5.46
Native T_7 DNA	Sodium acetate, pH 6.0	0.005	3.52	8.67	12.3
Native T_7 DNA	Sodium acetate, pH 6.0	0.10	1.49	4.30	6.56
Denatured T_7 DNA	Sodium acetate, pH 6.0	0.10	1.67	4.44	6.62
Native Qβ RF	Sodium acetate, pH 7.0	0.025	7.89	12.7	14.8
Denatured Qβ RF	Sodium acetate, pH 7.0	0.025	8.63	12.4	16.3
Native Qβ RF	Tris-acetate, pH 7.0	0.025	2.24	3.60	5.80
Denatured Qβ RF	Tris-acetate, pH 7.0	0.025	2.30	4.20	6.01
d.s. Reo RNA	Sodium acetate, pH 7.0	0.02	2.85	6.47	8.13
Denatured Reo	Sodium acetate, pH 7.0	0.02	3.20	6.95	8.54
d.s. Reo RNA	Tris-acetate, pH 7.0	0.02	1.14	2.54	3.22

[a] All reactions were incubated at 50° in the presence of an 8–10-fold molar excess of [^{203}Hg] mercuric acetate (1–2 × 10^7 cpm/μmol). The polymer concentrations used were: calf thymus DNA, 7 × 10^{-4} *M*; T_7 DNA, 4.4 × 10^{-4} *M*; Qβ replicative form, 6 × 10^{-4} *M*; Reo RNA, 1 × 10^{-4} *M*. All reactions were processed as described in the legend to Figure 6.

single-stranded polymers. The structure of duplex polynucleotides in the mercuration reactions is not, however, truly native. Noncovalent Hg^{2+}–polymer complexes are formed almost immediately upon addition of mercuric acetate. Although the binding of Hg^{2+} to native DNA (or RNA duplexes) does not induce complete polymer denaturation (Eichhorn and Shin, 1968; Nandi et al., 1965), the bound Hg^{2+} ions must cause a local denaturation or a distortion in the normal helical structure since the Hg^{2+}–base interactions involve amino groups and ring nitrogens (Yamane and Davidson, 1961) which are buried in the native structure. The true substrates in the mercuration of "native" polymers are most likely structurally modified Hg^{2+}–polymer complexes in which the sites for convalent modification are as readily accessible as those of single-stranded polynucleotides.

As shown in Figure 11, under similar reaction conditions ribosomal and R17 RNA react at about twice the rate of fd and T_7 DNA, although all polymers have approximately the same A + T (U) base composition. On the basis of the base specificity shown in Table V one would expect both U and C residues in RNA, but only C residues in DNA, to be modified. Analysis of the chemical and enzymatic degradation products of mercurated polymers confirms this expectation (Dale and Ward, 1975), provided the reaction times are not of extended duration (24 hr or longer). By increasing the mercuric acetate/nucleotide ratio to 30:1 (cf. 10:1 in Figure 11), quantitative modification of all reactible pyrimidine bases can be obtained within 8 hr. However, further incubation at 50° gives a slow but continual rise in the level of polymer bound mercury. Analyses of polymers incubated for 24 hr under such conditions reveal the presence of a new ^{203}Hg-labeled compound which comprises 3–5% of the total bound mercury and possesses electrophoretic properties similar to that expected for 8-mercuri-GMP. The nature of this minor product was not, however, characterized further.

Heteropolymer mercurations, like poly(U) reactions, proceed optimally at pH 6.0–7.0, although the reaction rates do not exhibit as striking a dependence on the buffer salt concentration. For example, a 20-fold decrease in buffer concentration increases the mercuration rate of calf thymus and T_7 DNAs by only twofold (Table VI). An interesting and unexpected salt effect was, however, observed while

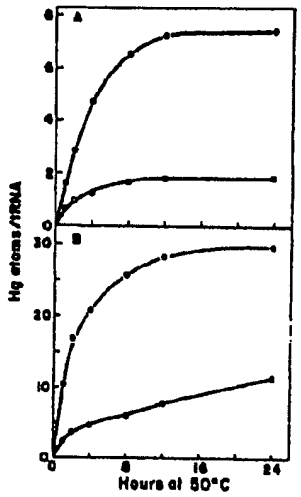

FIGURE 12: Mercuration of yeast phenylalanyl-tRNA (A) in 0.01 *M* (●) and 0.5 *M* (○) Tris-acetate buffer (pH 7.0) and (B) in 0.05 *M* (●) and 0.5 *M* (○) sodium acetate buffer (pH 7.0). The results shown are the average of triplicate determinations. Each reaction (1.0 ml) contained 4.8 OD_{260} (7 nmol) of tRNA (5.2 × 10^{-4} *M* total nucleotide) and [^{203}Hg]mercuric acetate (2.3 × 10^7 cpm/μmol), 2 × 10^{-3} *M* (A); 5 × 10^{-3} *M* (B). Aliquots (0.15 ml) were removed at the indicated times and chromatographed on 1 × 10 cm columns of Sephadex G-25 using quench buffer (see Table V) as the eluent; 0.4-ml fractions were collected and 25 μl counted in Aquasol to locate the tRNA. The peak fractions were pooled and dialyzed against TNE buffer (see Table V). The ^{203}Hg/tRNA ratio was determined as previously described (Figure 6).

studying the mercuration of tRNA. In sodium acetate buffers tRNA (both purified and unfractionated species from *E. coli* and yeast have been tested) undergoes extensive substitution with kinetics similar to those observed for other polymers. In contrast, reactions done in Tris-acetate buffers yield only a limited number of modified bases. Results obtained with yeast phenylalanyl tRNA are illustrated in Figure 12. From the nucleotide sequence data (RajBhandary and Chang, 1968) and the base specificity of the mercuration reaction, it is estimated that yeast $tRNA^{Phe}$ should possess 28 reactible pyrimidines (ribothymidine, pseudouri-

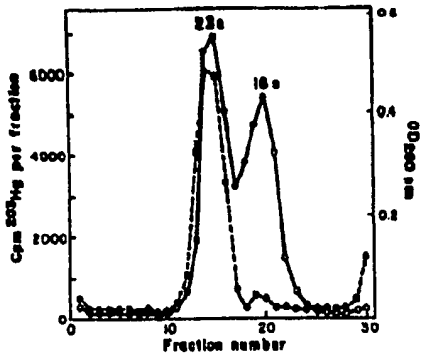

FIGURE 13: Sedimentation profile of 23S rRNA after mercuration of 18% of its pyrimidine bases. Five OD_{260} of 23S RNA was treated with [^{203}Hg]mercuric acetate for 90 min under the conditions given in Figure 11. The reaction was quenched and chromatographed on Sephadex G-25 as described in Table V to remove excess mercuric acetate. 0.1 ml of the RNA peak containing 0.15 OD_{260} (24,000 cpm of ^{203}Hg) was removed, 4.0 OD_{260} (50 μl) of cold (23S + 16S) rRNA added, and the sample centrifuged in a 5–20% sucrose gradient (in 0.01 *M* Tris-HCl buffer (pH 7.5)-0.1 *M* NaCl-10^{-3} *M* mercaptoethanol), for 2.5 hr at 48,000 rpm in an SW 50.1 rotor; 0.15-ml fractions were collected by bottom puncture, 50 μl was counted in 3.0 ml of Aquasol for ^{203}Hg cpm, and the residual sample was diluted to 1.0 ml with water for OD_{260} determinations.

dine, 5-methylcytidine, and dihydrouridine being excluded). Between 28 and 29 Hg atoms/tRNA was introduced when $tRNA^{Phe}$ was reacted for 12 hr in 0.01 *M* sodium acetate buffer (pH 7.0). Increasing the buffer concentration to 0.5 *M* decreased the rate significantly but the reaction does proceed slowly to essentially complete substitution. In contrast, the extent of substitution in 0.05 *M* and 0.5 *M* Tris-acetate buffer (pH 7.0) appears to be limited to about 7.2 and 2.0 Hg atoms/tRNA, respectively. The low level mercuration plateau in 0.5 *M* Tris-acetate buffer has been observed for all tRNA species examined; it occurs in the presence or absence of 10 m*M* Mg^{2+} ions, it is independent of temperature from 30 to 50°, and it is not significantly altered by increasing the Hg^{2+}/nucleotide ratio in the reaction mixture by 20-fold. Schmidt et al., 1973, recently reported that under certain conditions the thallic chloride catalyzed iodination of yeast $tRNA^{fMet}$ would preferentially occur on three cytidine residues; two in the amino acid acceptor stem and one in the anticodon loop. The possibility that a similar type of site specific reaction is occurring when tRNA is mercurated in Tris-acetate buffers is currently under investigation.

Limited mercuration of heteropolymers does not disrupt their structural integrity. The sedimentation profile of 23S rRNA is essentially unchanged after mercuration of 18% of the total pyrimidine bases (Figure 13). Reaction conditions which modify 8% of the C bases in the circular single-stranded fd DNA (1 hr at 50°) cause less than 15% of the molecules to undergo a single phosphodiester bond sisson. (We thank Dr. Gerald Bourguignon for analyzing the ratio of circular to linear DNA molecules in the electron microscope). The prolonged heating at 50° (7–8 hr) required for extensive or quantitative mercuration does, however, introduce considerable strand cleavage. Since the introduction of only a small number of mercury atoms is sufficient to give quantitative retention of mercurated polymers on sulfhydryl-Sepharose, structurally intact polymer probes can be prepared for use in the hybridization and selective fractionation procedure to be described (Dale and Ward, 1975).

Discussion

The mercurinucleotides described in this paper represent a new class of nucleotide analogs which have a number of unusual and potentially useful properties. Being organomercurial compounds, they do not possess the same degree of chemical stability as classical nucleotides although they are relatively stable in aqueous solutions free of excess reducing agents. Little, if any, cleavage of the mercury–carbon bond occurs under physiological conditions and only a few percent hydrolysis occurs after standing for 3–4 days at room temperature in 0.01 *M* HCl or 0.01 *M* NaOH. The compounds therefore appear to be sufficiently stable to be utilized as heavy atom derivatives for X-ray crystallographic studies. Indeed, the enzymatic incorporation of a single Hg-CMP residue into the amino acid acceptor stem of tRNA has been achieved (Darling, Dale, and Ward, unpublished results; P. Sigler, personal communication) and crystals of mercurated yeast $tRNA^{fMet}$ obtained (P. Sigler, personal communication). The high affinity of organomercurials for mercaptans (association constants of about 10^{14} compared to 10^3 for acetate, Simpson, 1961) makes the mercurinucleotides convenient "starter" molecules for the in situ synthesis of a variety of nucleotide mercurithioesters. These compounds can be used directly to probe some of the steric parameters of enzyme nucleotide binding sites. By such mercaptan manipulations we have observed that the nucleotide binding sites of template dependent DNA and RNA polymerases are sterically quite different from those of other polynucleotide binding proteins (Dale et al., 1973; Dale and Ward, unpublished results). These observations suggest that mercurinucleotides may have general utility as probes of both protein and polynucleotide structure.

Although sufficiently stable to permit routine biochemical studies, the mercury–carbon bond is extremely sensitive to cleavage by electrophiles and reducing agents. This necessitates certain precautions in their handling. For example, the presence of hydroquinones or other antioxidants in phenol, etc., will rapidly catalyze demercuration. Similarly, when utilizing mercaptan (or cyanide) ligands, the mercaptan/Hg ratio should be maintained as close to unity as experimentally feasible since a large mercaptan excess will also cause demercuration. The lability of the mercury–carbon bond to such agents can, however, be put to useful advantage. Treatment of mercurinucleotides (and mercurated polynucleotides) with I_2, *N*-bromosuccinimide, or [^{3}H]sodium borohydride has been found to rapidly generate the corresponding iodinated, brominated, and tritiated compounds (Dale, Livingston and Ward, manuscript in preparation). The tritiation or radioiodination of polynucleotides via mercuri intermediates is done under very mild conditions and produces no uracil hydrates in RNA. This method should have some utility in the preparation of radiolabeled oligo- and polynucleotides, particularly for enzyme binding or in situ hybridization studies. Some addition properties and applications of mercurated polymers are described in the following paper (Dale and Ward, 1975).

Acknowledgments

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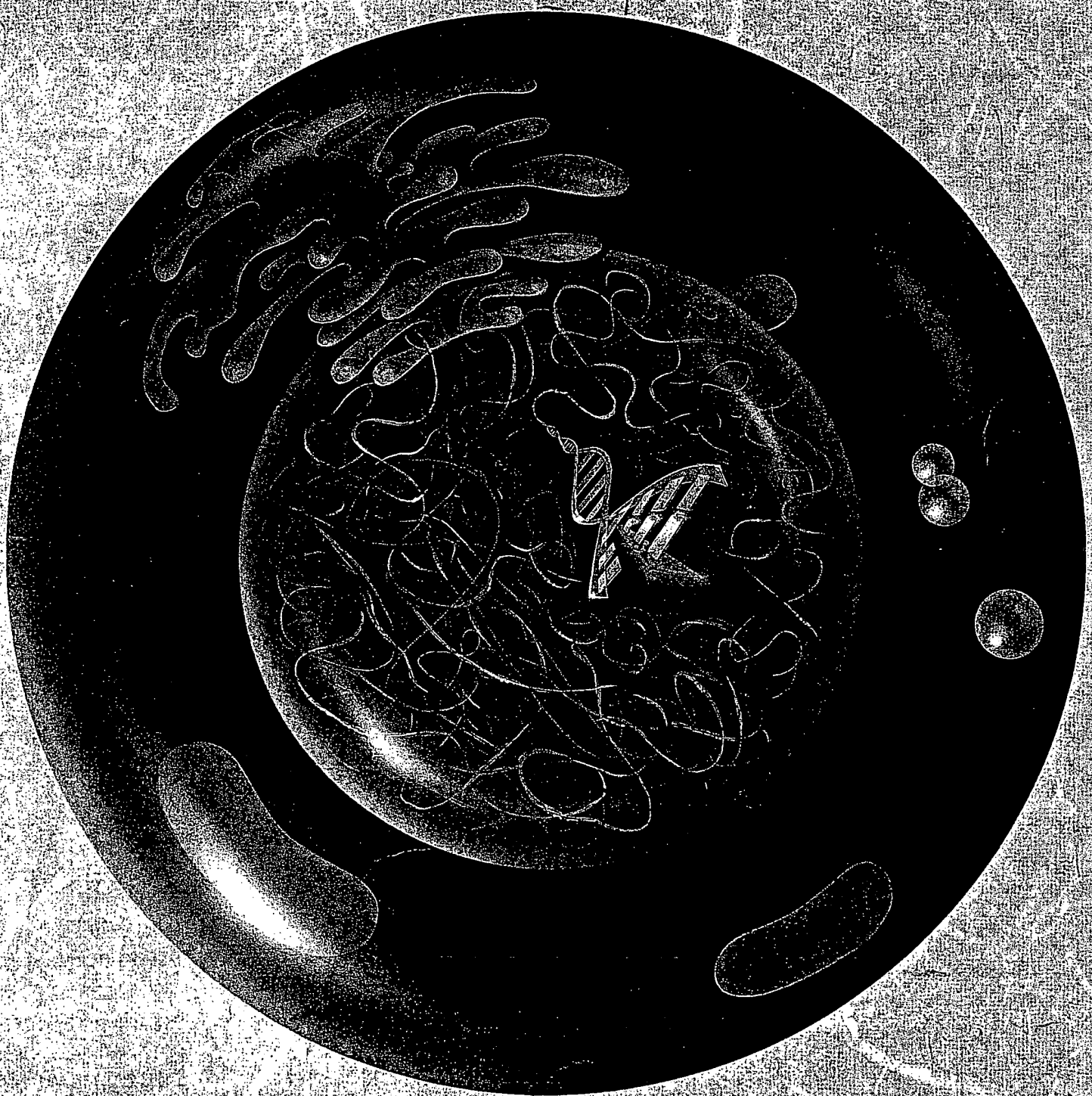

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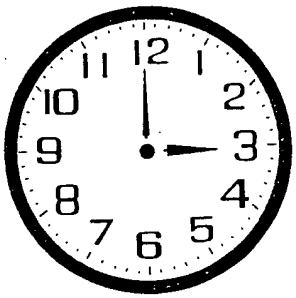

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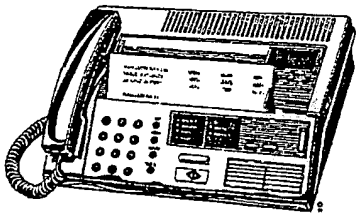

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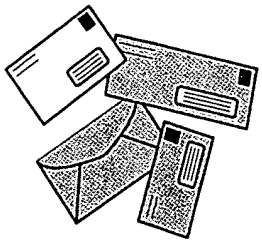

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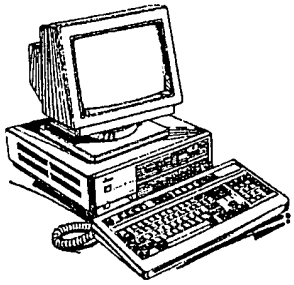

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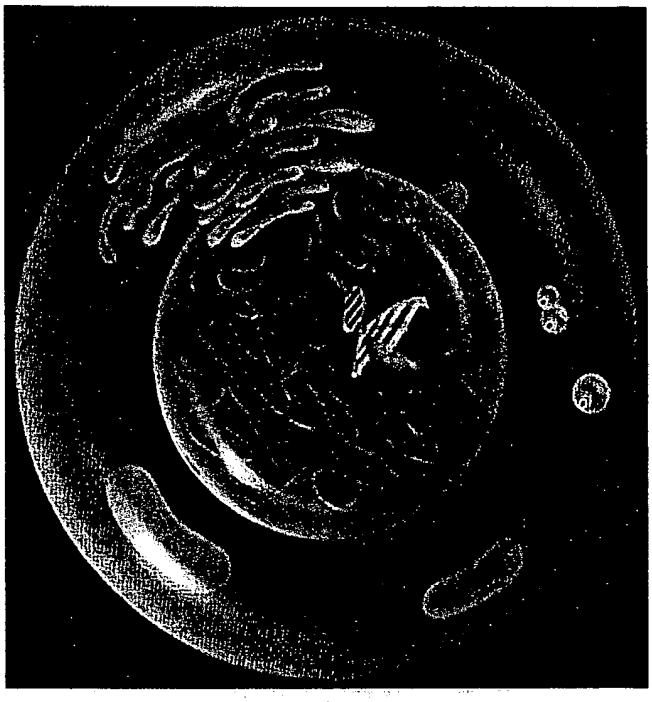

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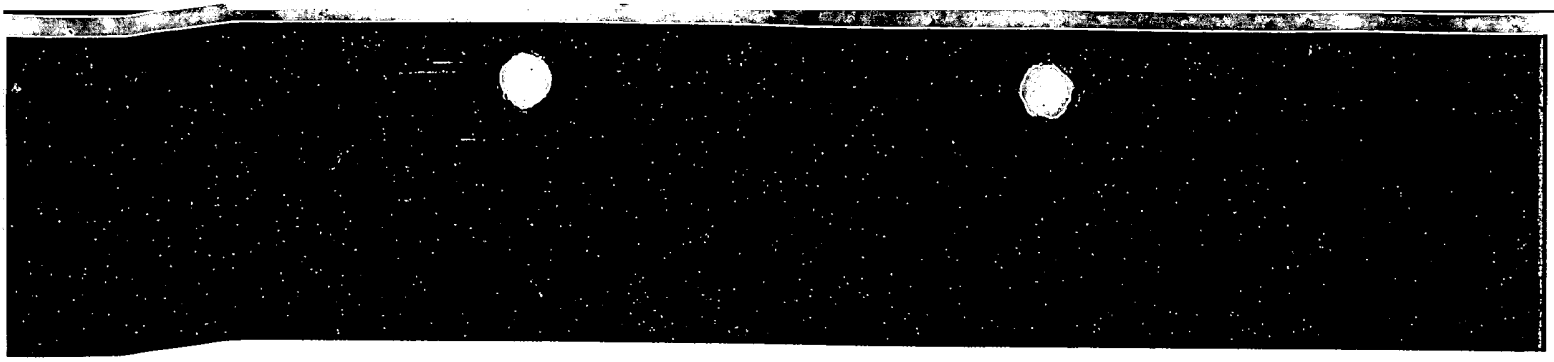

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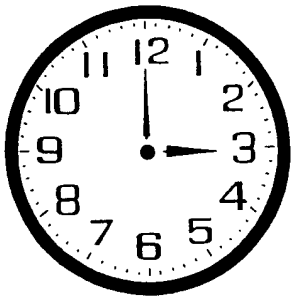

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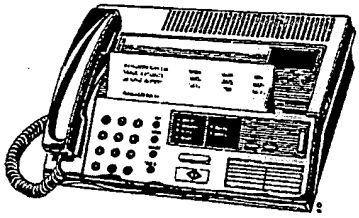

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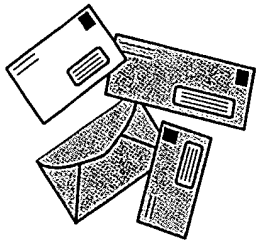

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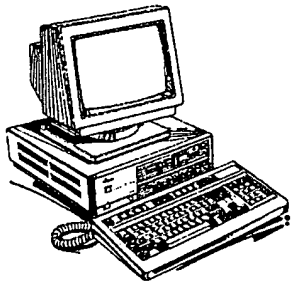

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Functional Genomics: Expression Analysis of *Escherichia coli* Growing on Minimal and Rich Media

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DNA arrays of the entire set of *Escherichia coli* genes were used to measure the genomic expression patterns of cells growing in late logarithmic phase on minimal glucose medium and on Luria broth containing glucose. Ratios of the transcript levels for all 4,290 *E. coli* protein-encoding genes (cds) were obtained, and analysis of the expression ratio data indicated that the physiological state of the cells under the two growth conditions could be ascertained. The cells in the rich medium grew faster, and expression of the majority of the translation apparatus genes was significantly elevated under this growth condition, consistent with known patterns of growth rate-dependent regulation and increased rate of protein synthesis in rapidly growing cells. The cells grown on minimal medium showed significantly elevated expression of many genes involved in biosynthesis of building blocks, most notably the amino acid biosynthetic pathways. Nearly half of the known RpoS-dependent genes were expressed at significantly higher levels in minimal medium than in rich medium, and *rpoS* expression was similarly elevated. The role of RpoS regulation in these logarithmic phase cells was suggested by the functions of the RpoS dependent genes that were induced. The hallmark features of *E. coli* cells growing on glucose minimal medium appeared to be the formation and excretion of acetate, metabolism of the acetate, and protection of the cells from acid stress. A hypothesis invoking RpoS and UspA (universal stress protein, also significantly elevated in minimal glucose medium) as playing a role in coordinating these various aspects and consequences of glucose and acetate metabolism was generated. This experiment demonstrates that genomic expression assays can be applied in a meaningful way to the study of whole-bacterial-cell physiology for the generation of hypotheses and as a guide for more detailed studies of particular genes of interest.

The field of microbial physiology was launched in 1958 with the fundamental discovery that the macromolecule composition of the bacterial cell changes with the growth rate (58). Faster-growing cells contain proportionally more stable RNAs—rRNA and tRNA. The reason for this increased abundance of stable RNA is simple: in order to grow faster, bacteria must synthesize protein faster. The growth rate of the bacterial cell increases in proportion to the quality of the growth medium (although not necessarily in proportion to its exact composition), and this increase in growth rate is accomplished by an increase in the number of ribosomes and the concentrations of translation accessory factors (8). It is now understood that the seven *Escherichia coli* rRNA operons are under the control of growth rate-dependent promoters and that expression of the ribosomal proteins, translation factors, and the transcription apparatus are all tied to the cellular concentration of rRNA (8, 27, 35). The rate of transcription initiation of the growth rate-dependent *rrn* promoters is physiologically connected to the metabolic state of the cell by the concentration of nucleoside triphosphates—efficient transcription initiation from these promoters requires a high concentration of the initiating nucleotide (22). The presence of high-quality nutrients in the growth medium results in high intracellular nucleoside triphosphate concentrations; hence, this model unifies the idea that the quality of the growth medium dictates the growth rate of the cell.

Growth rate-dependent changes in cell composition are realized at the level of gene expression; for example, transcript levels corresponding to the protein components of the protein synthesis apparatus change in proportion to the growth rate as the rates of transcription or mRNA turnover are modulated (27, 35). Other changes in cellular physiology can be more subtle, such as redirection of intermediary metabolism in response to changes in growth medium composition or the flow of carbon and electrons that is coupled to ATP generation, although many of these adjustments in metabolism are accompanied by changes in the concentrations of metabolic enzymes and electron transport chain components (40, 41, 56, 63, 64). The expression of numerous other genes is affected by environmental stresses (9, 17, 26, 29, 48, 60, 69, 71). Almost all aspects of microbial physiology, including the myriad adjustments made by the cell in response to changes in the environment, have been cataloged by the scientific community in the form of the book *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Since the publication of this compendium, the sequence of the *E. coli* genome has been completed and the way that we look at gene expression is forever changed (6). The genome sequence provides the tools necessary to take a global view of *E. coli* physiology.

Genomic expression assays provide an unprecedented ability not only to look at a single aspect of physiology but also to see how a particular gene, regulon, or modulon interacts with every other aspect of physiology. Genomewide methods have been developed for a number of uses, including drug discovery (43), measurement of gene copy number (50), discovery of disease-related genes in humans (18, 28), gene mapping (12), and gene expression: in humans (73), in yeast (13, 19, 31, 37, 65), and in *Arabadopsis* (59).

From the *E. coli* MG1655 genome sequence (6), 4,290 open

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reading frame (ORF)-specific primer pairs were designed for PCR amplification of all *E. coli* ORFs, and this set of 4,290 PCR-amplified, ORF-specific DNA fragments was used to develop DNA arrays for gene expression profiling (54). A similar set of ORF-specific DNA fragments was used to generate commercially available DNA macroarrays (12 by 24 cm) on nylon membranes (Sigma-GenoSys Biotechnologies, Inc., Woodland, Tex.). The advantage of the commercial arrays is that they can be used with equipment found in typical molecular biology laboratories. For these utilitarian investigations of bacterial physiology to be successful, it will be necessary to determine if DNA macroarrays can reveal differences in gene expression across the genome. Here we report on the expression profiles of *E. coli* under two very different growth conditions, and from the data we provide insights into growth rate-dependent gene expression, global regulation of biosynthetic regulons, and stress responses that appear to be involved in growth on minimal glucose medium.

MATERIALS AND METHODS

Growth conditions. *E. coli* MG1655 cultures were grown in 50-ml batch cultures in 250-ml Erlenmeyer flasks at 37°C with aeration by gyrotary shaking (300 rpm). The culture media used were M63 minimal medium (57) containing 0.2% glucose and a rich medium, Luria broth (39) containing 0.2% glucose. Growth was monitored spectrophotometrically at 600 nm on a Spectronic 601 (Milton Roy). Cells were harvested in late logarithmic growth phase (absorbance at 600 nm = 0.6) from cultures that had been inoculated at low density and had maintained a constant growth rate for at least 10 generations.

Handling of RNA. The ability to isolate pure, intact mRNA is critical to the success of genomic expression assays. Cells in growing cultures were pipetted directly into boiling lysis buffer. The lysed cells were extracted twice with phenol (pH 5.0) at 60°C and then with phenol-chloroform (66). The RNA was precipitated with isopropanol, redissolved in water, treated with DNase I, and applied to an RNeasy column. The purified RNA was redissolved in water and stored at −70°C in 2 volumes of ethanol.

Probe synthesis. Hybridization probes were generated by standard cDNA synthesis. The protocol supplied by the manufacturer of the DNA arrays was suitable for achieving >70% incorporation of the ^{33}P-labeled nucleotide. Since it is not possible to purify bacterial mRNA from total RNA (i.e., by purification of polyadenylated mRNA as in eukaryotes), the labeling protocol takes into account the presence of rRNA and tRNA, which constitute 85% of the total RNA. The C-terminal primer set (4,290 ORF-specific C-terminal primers [Sigma-GenoSys Biotechnologies, Inc.]) was used to generate the hybridization probe in a standard first-strand cDNA synthesis. Briefly, 1 μg of RNA was mixed with dATP, dGTP, and dTTP (final concentrations, 0.33 mM each), and cDNA-labeling primers (Sigma-GenoSys), in a volume of 25 μl of first-strand buffer, heated to 90°C for 2 min and cooled to 42°C in 20 min. Then 200 U of Superscript II, 10 U of RNase inhibitor, and 20 μCi of [α-^{32}P]dCTP (2,000 to 3,000 Ci/mmol) were added, bringing the total volume to 30 μl, and the cDNA synthesis reaction mixture was incubated at 42°C for 2 h. Unincorporated nucleotides were removed by gel filtration through a G-50 Sephadex column (57).

Hybridization. The DNA arrays (Panorama *E. coli* gene arrays) used in the hybridization experiments were produced by Sigma-GenoSys Biotechnologies, Inc. Each DNA array consists of a 12- by 24-cm positively charged nylon membrane on which 10 ng each of all 4,290 PCR-amplified ORF-specific DNA fragments are robotically printed in duplicate. The hybridization and washing steps were carried out as described by the manufacturer. Briefly, the blots were prehybridized in hybridization solution (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7], 2% sodium dodecyl sulfate [SDS], 1× Denhardt's reagent, 100 μg of sheared salmon sperm DNA per ml) at 65°C for 1 h in a 30- by 3.5-cm roller bottle in a hybridization oven. The entire cDNA probe, generated as described above, was added to 3 ml of hybridization buffer, and the blot was hybridized with this solution for 15 h at 65°C. The blots were washed with buffer (0.5× SSPE, 0.2% SDS) three times for 5 min each at room temperature and three times for 30 min each at 65°C. The blots were then wrapped in clear plastic food wrap and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, Calif.) for 48 h. For each of the data sets used in this study, the same blot was consecutively hybridized, stripped, and rehybridized (this can be done up to four times). The blots were stripped at 100°C with 1% SDS in Tris-EDTA buffer as specified by the manufacturer.

Data analysis. The exposed PhosphorImager screens were scanned with a pixel size of 100 μm (10,000 dots/cm^2) on a STORM 840 PhosphorImager (Molecular Dynamics). The resulting TIFF image files were analyzed by determining the pixel density (intensity) for each spot in the array by using ImageQuant (version 5.0) software (Molecular Dynamics). A grid of individual ellipses corresponding to each of the DNA spots on the blots was laid down on the image to designate each spot to be quantified. Background was subtracted automatically by the software by using the local median background subtraction method. The intensities for each spot were exported from ImageQuant into a Microsoft Excel spreadsheet. Each ORF-specific spot was present in duplicate, and the intensities were averaged for analysis. Each averaged spot intensity was expressed as a percentage of the total of intensities of all the spots on the DNA array, which allowed direct comparison of the two conditions by normalizing with regard to the specific activity of the probes used. The correlation coefficients of the percent intensities determined individually for the duplicate spots on a single blot ranged from 0.986 to 0.999, and the standard deviations for the log ratios of intensities of the duplicate spots (determined as described below) ranged from 0.073 to 0.095 for four different hybridizations, thus providing a measure of reproducibility.

Two growth conditions were compared by determining the ratio of the corresponding averaged percent intensities of each pair of ORF-specific spots on the two blots. These ratios represent the relative transcript levels of each *E. coli* ORF under the two growth conditions. Ratios were calculated such that the log of the absolute value of the expression ratio was positive for percent intensities that were higher under the first condition and negative for percent intensities that were higher under the second condition. Also taken into account in the calculation were situations where the percent intensities for both conditions fell below a threshold value equal to the background, that is, when the gene was not expressed at detectable levels under either condition; in this case, the calculated log expression ratio was zero. A threshold value, equal to the background, was used to calculate ratios where a gene was not expressed at detectable levels under one of the growth conditions. A statistical analysis of the log expression ratios of all 4,290 genes in the minimal glucose versus gluconate experiment indicated a standard deviation from the mean (0.000) of 0.180. There is 95.5% confidence that any expression ratio is significant if the value of the log expression ratio is greater than 2 standard deviations (0.360) from the mean. Thus, a log expression ratio of 0.400 (2.5-fold) was considered to indicate significantly higher expression (99% confidence of each tail) in the analyses, and this value is shown graphically in Fig. 3 to 6. The experiment presented here, comparing the expression profile of cells grown on minimal versus rich medium, was repeated, and qualitatively similar data were obtained (data not shown). The blot-to-blot reproducibility of DNA macroarray hybridization data has been addressed in detail elsewhere (54).

Functional groups. Two schemes for functional grouping of genes have been applied to the expression data generated in these experiments. The first scheme assigns genes to groups in accordance with their cellular function, as described previously (6). The second scheme of functional assignments is that of Riley (55), version M54, submitted by Plunkett et al. (19a), as it appears on the *E. coli* K-12 MG1655 complete genome at the National Center for Biotechnology Information (43a).

Internet access to data. An Internet accessible version of the expression data and details of the protocols has been created (49a). The data can also be accessed from a database (19a).

Chemicals. SuperScript II, an RNase H$^-$ reverse transcriptase used for cDNA synthesis, was purchased from Gibco BRL (Bethesda, Md.). RNase inhibitor and DNase I were also purchased from Gibco BRL. PCR grade deoxyribonucleoside triphosphates were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.). RNeasy columns were purchased from Qiagen, Inc. (Valencia, Calif.). [α-^{33}P]dCTP (2,000 to 3,000 Ci/mmol) was purchased from New England Nuclear (Wilmington, Del.). Biochemicals were purchased from Sigma (St. Louis, Mo.).

RESULTS AND DISCUSSION

The genomic expression profiles of *E. coli* MG1655 growing on rich and on minimal culture media (Fig. 1) were determined. The rich medium (Luria broth) contained amino acids as the nitrogen source, a number of other preformed building blocks of macromolecule synthesis (e.g., nucleosides and vitamins, etc., provided by tryptone and yeast extract), and also glucose as a carbon and energy source. The minimal medium contained glucose as the sole carbon and energy source and ammonia as the nitrogen source. In glucose minimal medium, the carbon backbone of the glucose molecule was rearranged through the biosynthetic pathways to generate each of the building blocks de novo. In addition to having fundamentally different metabolisms, the two cultures grew at significantly different rates: G = 25 min on the rich medium and G = 57 min on minimal glucose medium. As a control, data are provided for a culture growing on minimal gluconate medium (G = 60 min).

Whole-genome perspective. RNA isolated from the cultures in Fig. 1 were used to generate the probes used for hybridization of the DNA arrays shown in Fig. 2, and the data were

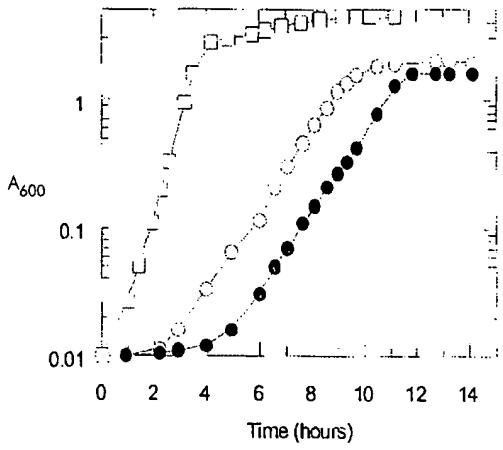

FIG. 1. Growth of *E. coli* MG1655 on Luria broth plus glucose (open squares), minimal glucose medium (open circles), and minimal gluconate medium (solid circles). Cells were harvested for genomic expression analysis at an absorbance at 600 nm (A_{600}) of 0.6.

quantified as described in Materials and Methods. Calculation of the log expression ratios of corresponding spots allowed pairwise comparisons of the relative transcript levels for each of the 4,290 *E. coli* protein-encoding genes under the different growth conditions. The log expression ratios indicate whether gene expression is higher under one condition or the other or remains unchanged. The results are summarized in Table 1 and presented in chart form in Fig. 3 to 6. It is important to keep in mind that in vivo transcript levels are dynamically balanced by the rates of transcription initiation and transcript turnover. Thus, the data presented here as expression ratios reflect the relative transcript levels for individual genes without providing any indication of the mechanism of regulation. Furthermore, some individual expression ratios may be in error, due to technical problems, including cross-hybridization, PCR failures, misapplied DNA spots on the arrays, or scatter in the data (see reference 54 for a more comprehensive review of the technical aspects of using *E. coli* DNA arrays). A few of the ratios are in conflict with published results, and it is possible that other ratios will not be validated in subsequent experiments. Thus, these data should not be taken as specific evidence for gene regulation and should be independently verified. Nevertheless, the general trends of the data are substantially clear and will be of value for generating experimental leads.

Expression levels of the majority of genes did not differ significantly (log ratio ≥ 0.4) between growth conditions. This was particularly true for the comparison of the cultures grown on minimal glucose versus minimal gluconate media; 80 genes (1.9%) were expressed at significantly higher levels on glucose, and 82 genes were expressed at significantly higher levels on gluconate (Table 1; Fig. 3). Thus, the overall similarity of these two growth conditions, being identical in basal medium composition, aeration, pH, and temperature and differing only in the nature of the carbon source, was reflected in their gene expression profiles. The comparison of genomic expression patterns of cells grown on minimal versus rich media was more revealing: 225 genes (5.2%) were expressed at significantly higher levels on minimal glucose, and 119 genes (2.8%) were expressed at significantly higher levels on rich medium (Fig. 3). A larger number of genes (3,496 versus 3,284 genes) had expression intensities above the background value on minimal glucose compared to rich medium (data not shown). By these measures, the cells growing on glucose minimal medium expressed more genes than did cells growing on rich medium. The nature of these differences in global gene expression was examined in detail, as described below.

Translation apparatus. The culture containing rich medium plus glucose grew more than twice as fast as did the cultures on minimal media (Fig. 1). It is known that faster-growing cells synthesize protein faster and contain more ribosomes (27, 35). There are 128 known genes encoding the enzymes, factors, and structural components that make up the translation apparatus. Of these 128 genes of the translation apparatus, 53 (41.4%) were expressed at significantly higher levels in the cells growing on rich medium and none of them were expressed at significantly higher levels on the minimal medium. Of the 53 translation genes that were expressed at higher levels on rich medium, 42 encoded ribosomal proteins. These data are charted in Fig. 4 and can be compared to the data for the cultures on minimal glucose versus gluconate medium, which had nearly identical growth rates and showed very few significant differences in expression of the translation genes. A comparison of the general pattern of expression of the translation genes (Fig. 4) to that of the entire *E. coli* gene set (Fig. 3) further illustrates the dramatic increase in production of the translation apparatus in the faster-growing cells.

(i) tRNA synthetase genes. There are 37 known genes encoding the tRNA synthetases and other enzymes involved in tRNA modification. While none of the expression ratios of the tRNA synthetase genes varied significantly, it is clear from the chart in Fig. 4 that the transcript levels for these genes followed the same general trend as the complete set of translation genes. This result is consistent with the notion that synthesis of the tRNA synthetases is coupled to the synthesis of other ribosomal components (27).

(ii) Translation factors. There are 17 known genes that encode factors involved in translation and ribosome modification, including the initiation and elongation factors, and 7 of these genes were expressed at significantly higher levels on rich medium (Fig. 4; Table 2). This result is generally consistent with the coupled synthesis of translation factors and ribosome components (27). The expression ratio of *infB* was significantly higher on rich medium. The regulation of *infB*, which is downstream of and cotranscribed with the transcription factor gene *nusA*, is complex and is thought to be the result of autoregulation of the extent of readthrough at upstream terminators by NusA (27). The expression ratio of *infB* was 1.8-fold higher than that of *nusA* (data not shown). The expression ratios of the translation elongation factor genes *tsf*, *tufB*, *tufA*, and *fusA* were all significantly higher, in that order, on rich medium, which is consistent with their coordinate regulation with the ribosomal protein genes (27). The growth rate-dependent regulation of *tsf*, *tufA*, and *fusA*, all of which are located in ribosomal protein operons, is the result of mRNA destabilization in slowly growing cells (27). Interestingly, regulation of *tufB* appears to be at least partially dependent upon Fis (68), and the *fis* gene had one of the highest expression ratios on rich medium, as described in more detail below. A fifth elongation factor encoded by *efp* has been shown to be essential in *E. coli* for protein synthesis and viability, although the details of *efp* regulation have not been published (2). The results of this study indicate that *efp* was expressed at a significantly higher level (log ratio = −0.425) in the faster-growing cells on rich medium, paralleling the expression of the other elongation factors.

(iii) Ribosomal proteins. Of the 55 genes encoding the ribosomal proteins, 42 were expressed at significantly higher

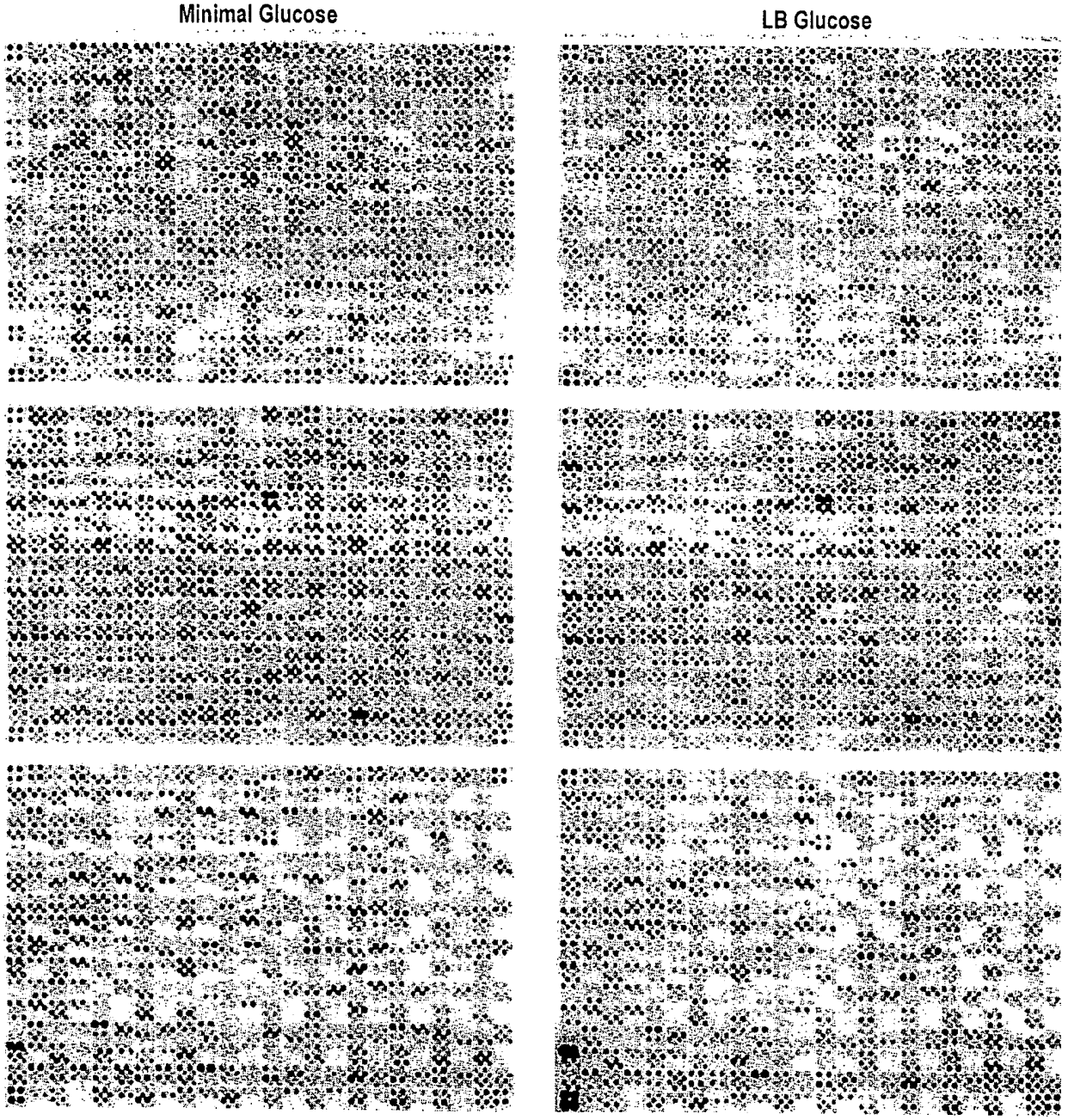

FIG. 2. DNA arrays of the entire set of *E. coli* genes hybridized with probes generated from RNA extracted from cells growing in late logarithmic phase on minimal glucose medium (left) and on Luria broth (LB) containing glucose (right).

levels in the more rapidly growing cells in rich medium (Fig. 4; Table 2). This result is consistent with the paradigm of growth rate-dependent regulation of ribosome number (35). Although the ribosomal S10 operon is at least partially regulated at the transcriptional level, it is generally accepted that regulation of the 21 ribosomal protein operons is not at the level of transcription initiation (23, 35). Rather, the regulation of ribosomal protein synthesis involves a combination of translational control and transcriptional control at the level of mRNA stability. In general, growth conditions which lead to a decreased rate of ribosome synthesis result in an excess of ribosomal proteins, with certain ones serving as autoregulators by binding to their transcript and decreasing the translation rate of the mRNA, thus leading to destabilization of the transcript (35). While not all of the ribosomal protein operons have been studied at this level of detail, the experiment presented here indicates that most of the operons are regulated in such a way that their transcript levels are higher in faster growing cells. Clearly, these data demonstrate that any regulatory mechanism that contributes to the dynamic control of a particular mRNA concentration, whether it be the rate of transcription or the rate of turnover, can be visualized in genomic expression

TABLE 1. Expression ratios of functional groups

Functional group	No. of genes[a]				
	Total	Minimal glucose vs Luria broth plus glucose		Minimal glucose vs minimal gluconate	
		Higher on minimal	Higher on LB	Higher on glucose	Higher on gluconate
Whole genome	4,290	225	119	80	82
Amino acid biosynthesis	97	22	0	3	0
Biosynthesis of cofactors, prosthetic groups, and carriers	106	9	1	0	2
Carbon compound catabolism	124	3	0	1	2
Cell processes	170	19	2	5	1
Cell structure	85	2	0	8	0
Central intermediary metabolism	149	15	1	4	5
DNA replication, repair, restriction/modification	105	1	0	1	1
Energy metabolism	136	14	5	4	3
Fatty acid and phospholipid metabolism	41	2	7	0	0
Hypothetical, unclassified, unknown	1,428	43	26	10	30
Nucleotide biosynthesis and metabolism	66	6	5	0	3
Phage, transposon, or plasmid	91	5	1	0	9
Putative cell structure	43	1	0	2	0
Putative enzymes	453	12	8	7	4
Putative factors	67	3	0	3	0
Putative membrane proteins	54	4	0	1	0
Putative regulatory proteins	167	11	0	4	1
Putative transport proteins	291	14	3	2	8
Regulatory function	208	14	3	6	3
Transcription, RNA processing, and degradation	28	0	1	0	1
Translation and posttranslational modification	128	0	53	6	1
Transport and binding proteins	254	24	2	13	8

[a] Number of genes showing significant (99% confidence) log expression ratios ($\geq \pm 0.400$).

assays. The global regulation and coordination of ribosome number and components of the translation apparatus was the most obvious result of this experiment.

Nitrogen metabolism. The minimal medium used in this study contained ammonia as the nitrogen source and the rich medium contained amino acids as the nitrogen source. In general, cells growing on minimal medium are limited for amino acids while cells growing on rich medium are limited for nucleotides (47, 52, 76). These differences were reflected in the transcript levels of the genes involved in nitrogen assimilation

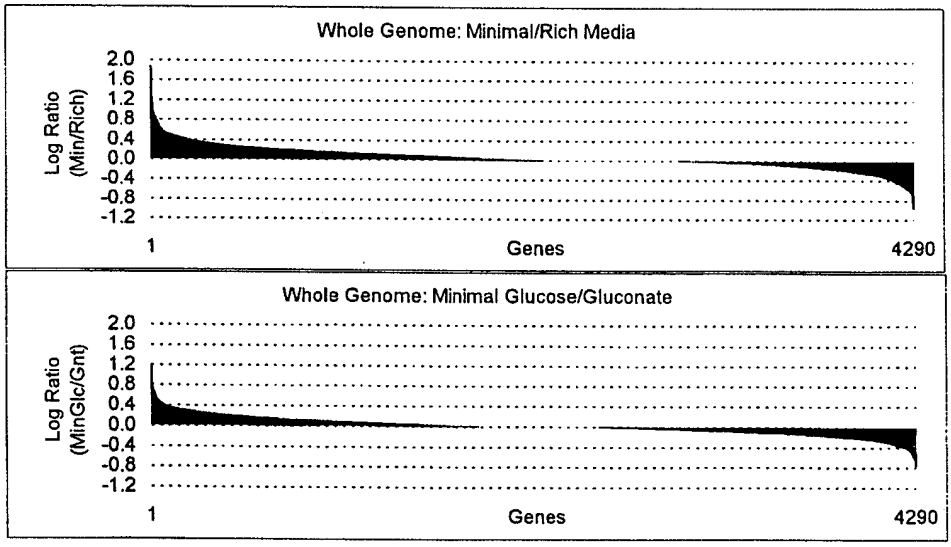

FIG. 3. The log expression ratios of all *E. coli* genes were plotted for minimal glucose versus Luria broth plus glucose (top) and for minimal glucose versus minimal gluconate (bottom). The entire data sets were sorted in Excel spreadsheets by the log expression ratio values, and a bar chart was generated by the software, with individual genes plotted on the *x* axis and the log expression ratios plotted on the *y* axis. Genes more highly expressed under the first condition are positive, and genes more highly expressed under the second condition are negative. The horizontal divisions (dashed lines) represent 99% confidence levels, such that any gene with a value extending beyond the first horizontal division in either direction is significantly expressed at a higher level under that condition.

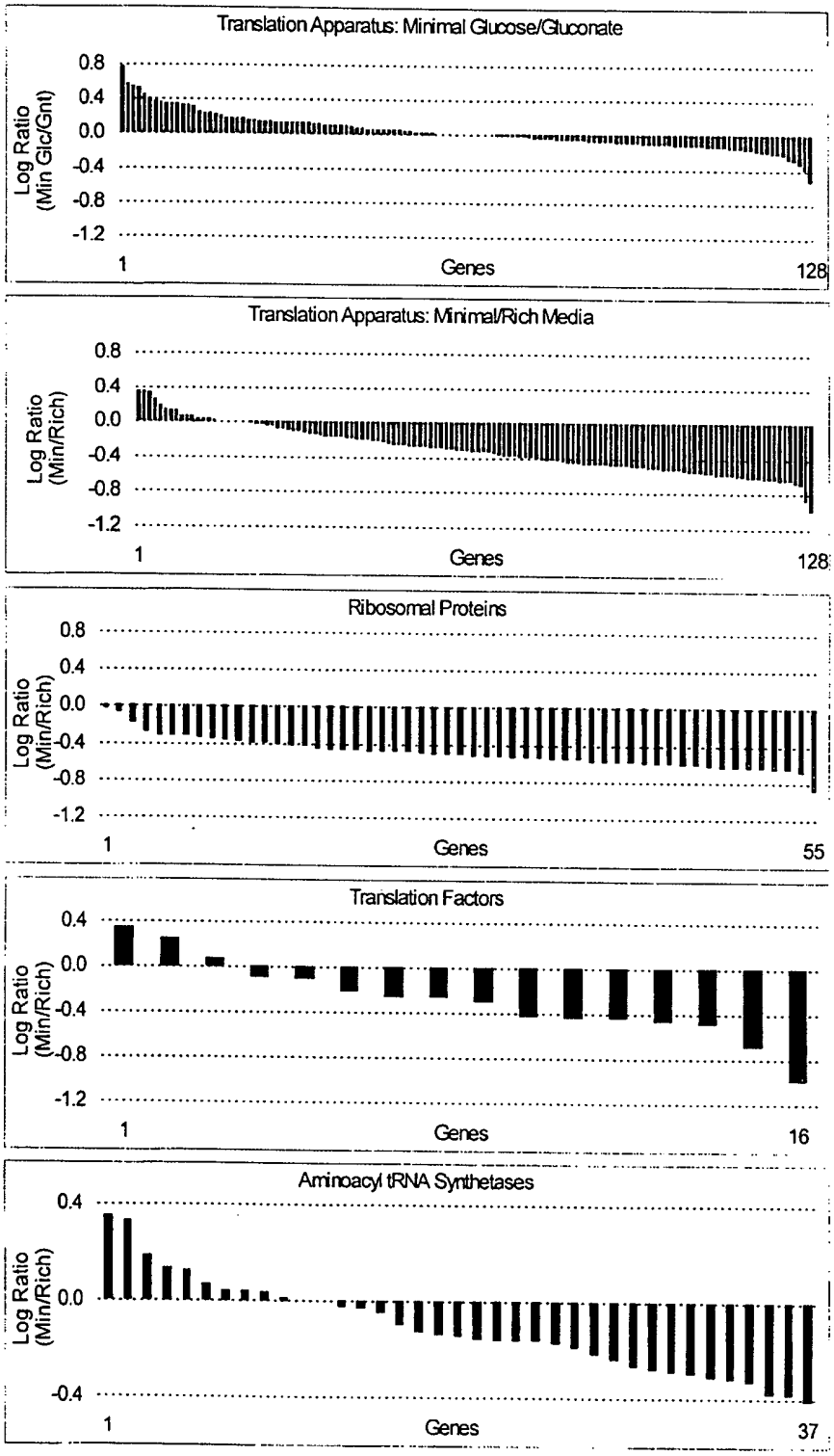

FIG. 4. Log expression ratios of the translation apparatus genes sorted by value. The set of all translation apparatus genes is shown in the top two panels for the minimal glucose versus minimal gluconate and minimal glucose versus Luria broth plus glucose experiments (see the legend to Fig. 3). The bottom three panels show the results of the minimal glucose versus Luria broth plus glucose experiment for functionally grouped subsets of the translation apparatus genes.

TABLE 2. Genes of the translation apparatus showing significant expression ratios

Gene	Gene product	Log ratio (minimal/rich medium)[a]
rplY	50S ribosomal subunit protein L25	−0.405
ileS	Isoleucine-tRNA synthetase	−0.414
rpsU	30S ribosomal subunit protein S21	−0.416
rpmH	50S ribosomal subunit protein L34	−0.419
efp	Elongation factor P	−0.425
fusA	GTP-binding protein chain elongation factor G	−0.435
tufA	Protein chain elongation factor Tu	−0.441
slyD	FKBP-type peptidylprolyl *cis-trans* isomerase	−0.450
rplL	50S ribosomal subunit protein L7/L12	−0.452
rpsO	30S ribosomal subunit protein S15	−0.461
rpmA	50S ribosomal subunit protein L27	−0.462
infB	Protein chain initiation factor 2	−0.463
rpmD	50S ribosomal subunit protein L30	−0.466
rpmE	50S ribosomal subunit protein L31	−0.471
rpsN	30S ribosomal subunit protein S14	−0.471
rplD	50S ribosomal subunit protein L4, regulates S10 operon	−0.475
prfB	Peptide chain release factor 2	−0.481
rpsD	30S ribosomal subunit protein S4	−0.483
rplF	50S ribosomal subunit protein L6	−0.494
rpsG	30S ribosomal subunit protein S7, initiates assembly	−0.499
ppiA	Peptidylprolyl *cis-trans* isomerase A	−0.501
rpsI	30S ribosomal subunit protein S9	−0.503
rpsK	30S ribosomal subunit protein S11	−0.504
rplK	50S ribosomal subunit protein L11	−0.514
rpmB	50S ribosomal subunit protein L28	−0.518
rpmC	50S ribosomal subunit protein L29	−0.522
rplA	50S ribosomal subunit protein L1, regulates L1 and L11	−0.529
rpsL	30S ribosomal subunit protein S12	−0.530
rpsB	30S ribosomal subunit protein S2	−0.540
rplS	50S ribosomal subunit protein L19	−0.548
rplN	50S ribosomal subunit protein L14	−0.553
rplC	50S ribosomal subunit protein L3	−0.555
rplP	50S ribosomal subunit protein L16	−0.570
rplU	50S ribosomal subunit protein L21	−0.575
rplQ	50S ribosomal subunit protein L17	−0.577
rplV	50S ribosomal subunit protein L22	−0.580
rpsT	30S ribosomal subunit protein S20	−0.587
rplE	50S ribosomal subunit protein L5	−0.587
rplR	50S ribosomal subunit protein L18	−0.596
rplM	50S ribosomal subunit protein L13	−0.603
rplI	50S ribosomal subunit protein L9	−0.607
prmA	Methylase for 50S ribosomal subunit protein L11	−0.615
rpsA	30S ribosomal subunit protein S1	−0.620
rplW	50S ribosomal subunit protein L23	−0.627
rpsS	30S ribosomal subunit protein S19	−0.634
rplB	50S ribosomal subunit protein L2	−0.636
rpsR	30S ribosomal subunit protein S18	−0.637
rpsJ	30S ribosomal subunit protein S10	−0.644
rpsE	30S ribosomal subunit protein S5	−0.646
rplJ	50S ribosomal subunit protein L10	−0.676
tufB	Protein chain elongation factor Tu	−0.688
rplX	50S ribosomal subunit protein L24	−0.875
tsf	Protein chain elongation factor Ts	−0.990

[a] Log expression ratios of measured transcript levels determined for the two cultures. The log expression ratio is positive for genes that were more highly expressed on minimal glucose medium and negative for genes that were more highly expressed on Luria broth plus glucose.

and biosynthesis of amino acids. The genes involved in assimilation of ammonia as the nitrogen source were expressed at significantly higher levels on minimal medium, including *gdhA*, which encodes glutamate dehydrogenase, and *gltD*, which encodes a subunit of glutamate synthase (Table 3). While it is known that *gdhA* is transcriptionally regulated by ammonia, next to nothing is known about the mechanism (53). The *gltBD* operon is subject to complex regulation by certain amino acids and in a concentration-dependent fashion by leucine-responsive protein (Lrp) (20); thus, the high induction ratio of *gltBD* on minimal medium (0.329 for *gltB*; 0.889 for *gltD*) can be explained by amino acid repression in rich medium and a high induction ratio of Lrp on minimal medium (see below). Conversely, *glnA*, which encodes glutamine synthase and is induced by nitrogen limitation (as indicated by a low ratio of intracellular glutamine to α-ketoglutarate), had the highest (although not significantly so) expression ratio (−0.316) of any of the amino acid biosynthetic genes in rich medium (52). In summary, the genes involved in ammonia assimilation were induced for growth on minimal medium where ammonia was the nitrogen source.

Biosynthesis of amino acids. The overall expression pattern of the genes encoding the enzymes of amino acid biosynthesis indicated that these were generally induced for growth on minimal medium (Fig. 5; Table 3). The *argA* gene, which encodes *N*-acetylglutamate synthase, the first enzyme of the pathway, and also *ygjG*, a probable ornithine aminotransferase, were expressed at significantly higher levels on minimal medium. Expression of the genes of the branched-chain amino acid biosynthetic pathways (67)was significantly elevated in minimal medium. The first gene of the *ilvGMEDA* operon, which encodes the enzymes of isoleucine and valine synthesis, was expressed at significantly higher levels on minimal medium. Interestingly, the monocistronic gene *ilvC*, which is derepressed exclusively by valine, had a log expression ratio of 0.977 on minimal medium, the highest of any of the amino acid biosynthesis genes. The leucine biosynthetic genes, encoded by the *leuABCD* operon, were all expressed at significantly higher levels on minimal medium. The high expression ratios of the leucine and valine biosynthetic genes are consistent with the relatively high abundance of these two amino acids (third and fourth most abundant, respectively) in *E. coli* cells (45). The genes encoding the first enzymes of the four branches of the aromatic amino acid biosynthetic pathways were all significantly elevated in cells grown on minimal medium (51). The first step of the "common pathway" of chorismate synthesis, encoded by *aroF*, and the first step of tyrosine biosynthesis, encoded by *tyrA*, form an operon, in that order, and had log expression ratios of 0.847 and 0.934, respectively. The *pheA* gene was significantly elevated, as were four of the five genes of the *trpEDCBA* operon; the *trpD* transcript level was high in both minimal and rich media. The gene encoding the first step in serine biosynthesis, *serA*, and the gene which codes for the enzyme that forms glycine from serine, *glyA*, were expressed at significantly higher levels on minimal medium. The *cysK* gene, which encodes cysteine synthase A, was expressed at significantly higher levels on minimal medium, while *cysM*, the gene encoding cysteine synthase B, was expressed at slightly higher levels on rich medium. The *cysE* product, serine transacetylase, forms a multifunctional complex with the *cysK* product, and the relative expression ratios of *cysK* and *cysE* (0.497 versus −0.024) are consistent with the *cysE* product being much less abundant in the enzyme complex (36). The uniquely MetR-regulated methionine synthase gene, *metE*, was expressed at a significantly higher level on minimal medium, in contrast to the remaining MetJ-regulated genes of methionine biosynthesis,

TABLE 3. Genes of nitrogen metabolism and biosynthesis showing significant expression ratios

Functional group	Gene	Gene Product	Log ratio (minimal/rich medium)
Amino acids	*ilvC*	Ketol-acid reductoisomerase	0.977
	leuD	Isopropylmalate isomerase subunit	0.951
	tyrA	Chorismate mutase-T and prephenate dehydrogenase	0.934
	gltD	Glutamate synthase, small subunit	0.889
	aroF	3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase	0.847
	leuA	2-Isopropylmalate synthase	0.809
	leuB	3-Isopropylmalate dehydrogenase	0.756
	serA	D-3-Phosphoglycerate dehydrogenase	0.633
	ygjG	Probable ornithine aminotransferase	0.623
	leuC	3-Isopropylmalate isomerase (dehydratase) subunit	0.566
	trpB	Tryptophan synthase, beta protein	0.563
	glyA	Serine hydroxymethyltransferase	0.541
	gdhA	NADP-specific glutamate dehydrogenase	0.519
	trpC	*N*-(5-Phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthetase	0.518
	ilvG1	Acetolactate synthase II, large subunit, interrupted	0.513
	trpE	Anthranilate synthase component I	0.511
	metE	Tetrahydropteroyltriglutamate methyltransferase	0.499
	cysK	Cysteine synthase A, *O*-acetylserine sulfhydrolase A	0.497
	aspC	Aspartate aminotransferase	0.482
	trpA	Tryptophan synthase, alpha protein	0.481
	argA	*N*-Acetylglutamate synthase	0.444
	pheA	Chorismate mutase P and prephenate dehydratase	0.439
	serC	3-Phosphoserine aminotransferase	0.401
Vitamins and cofactors	*nrdH*	Glutaredoxin-like protein; hydrogen donor	0.638
	hemC	Porphobilinogen deaminase	0.552
	entE	2,3-Dihydroxybenzoate-AMP ligase	0.543
	grxB	Glutaredoxin 2	0.506
	gst	Glutathionine *S*-transferase	0.501
	folE	GTP cyclohydrolase I	0.489
	ggt	Gamma-glutamyltranspeptidase	0.477
	entF	ATP-dependent serine activating enzyme	0.459
	entB	2,3-Dihydro-2,3-dihydroxybenzoate synthetase, isochorismatase	0.451
	entC	Isochorismate hydroxymutase 2 enterochelin biosynthesis	0.428
	thiH	Thiamine biosynthesis	−0.473
Nucleotides	*pyrI*	Aspartate carbamoyltransferase, regulatory subunit	0.628
	pyrB	Aspartate carbamoyltransferase, catalytic subunit	0.464
	prsA	Phosphoribosylpyrophosphate synthetase	−0.472
	ndk	Nucleoside diphosphate kinase	−0.484
	pfs	ORF, hypothetical protein	−0.515
	gmk	Guanylate kinase	−0.646
	upp	Uracil phosphoribosyltransferase	−0.646
Fatty acid and phospholipid metabolism	*cfa*	Cyclopropane fatty acyl phospholipid synthase	0.618
	fadA	3-ketoacyl-CoA thiolase	0.494
	fabA	β-Hydroxydecanoyl thioester dehydrase	−0.413
	fabD	Malonyl-CoA-[acyl-carrier-protein] transacylase	−0.437
	fabI	Enoyl-[acyl carrier protein] reductase	−0.468
	accC	Acetyl-CoA carboxylase, biotin carboxylase subunit	−0.557
	fabH	3-Oxoacyl-[acyl carrier protein] synthase III	−0.571
	fabF	3-Oxoacyl-[acyl carrier protein] synthase II	−0.576
	fabZ	(3*R*)-Hydroxymyristol acyl carrier protein dehydratase	−0.608

which did not vary significantly (25). The cobalamin-dependent methionine synthase encoded by *metH* was not expressed on minimal or rich media (data not shown). Overall, 8 of the 22 amino acid biosynthesis genes which were significantly elevated on minimal medium corresponded to the first step in the biosynthetic pathway. Thus, significant elevation of the first step in the amino acid biosynthetic pathways in cells grown on minimal medium was a recurring regulatory theme, consistent with the roles of these steps in controlling the flow of precursor metabolites out of the central pathways and into biosynthesis. Increased expression of the amino acid biosynthetic genes on minimal medium was indicative of the need to generate these building blocks from the sole carbon source, glucose.

Biosynthesis of vitamins, cofactors, prosthetic groups and carriers. Expression of the 106 genes involved in biosynthesis of vitamins, cofactors, prosthetic groups, and carriers followed the same trend as the genes of amino acid biosynthesis, although the expression ratios were generally not so large (Fig.

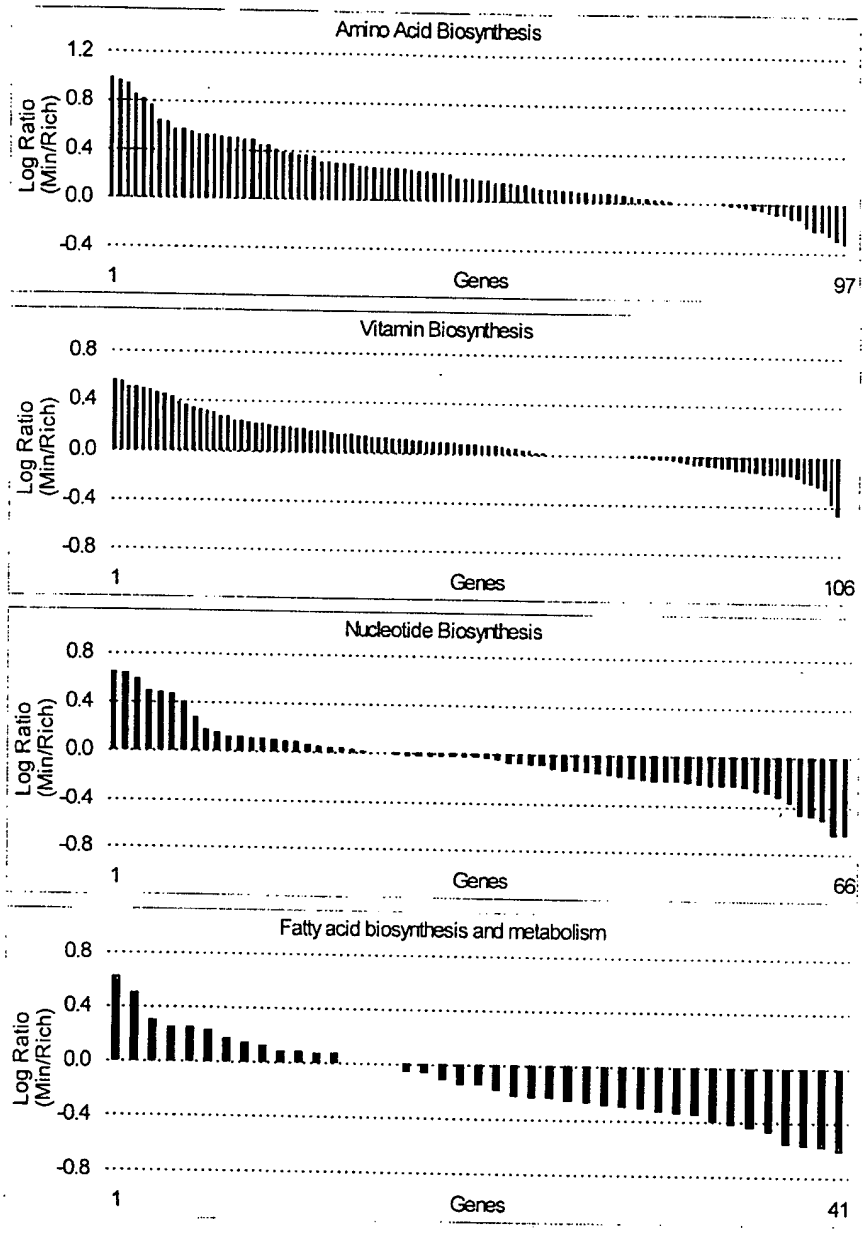

FIG. 5. Log expression ratios of biosynthetic genes were sorted by value for the minimal glucose versus Luria broth plus glucose experiment and are grouped by related pathways.

5; Table 3). Among the genes expressed at significantly higher levels on minimal medium were *hemC*, involved in porphyrin biosynthesis; the first three genes of the *entCEBA* operon and *entF*, involved in enterobactin biosynthesis; *grxB*, encoding glutaredoxin 2; *gst*, encoding glutathione *S*-transferase; *folE*, encoding the first step in tetrahydrofolate biosynthesis; and *ggt*, involved in glutathione biosynthesis. Only one gene, *thiH*, had an expression ratio that was significantly higher on rich medium, but this was in contrast to the remainder of the *thi* genes, which in general were expressed at modestly higher levels on minimal medium. Given that the vitamins and cofactors synthesized by the pathways in this functional group are needed in very small amounts, so small that they are rarely included in chemical composition tables (45), it is not surprising that most of these genes did not have significant expression ratios. Nevertheless, the general trend of higher expression in minimal medium is again indicative of the need to generate these building blocks de novo from glucose.

Nucleotide biosynthesis. While expression of the genes involved in biosynthesis of amino acids, vitamins, enzyme cofactors, and prosthetic groups, etc., was generally elevated on minimal glucose medium, expression of the genes involved in nucleotide salvage and biosynthesis was more evenly divided between the two growth conditions (Fig. 5; Table 3). The *pyrBI* genes, which form an operon encoding the first step of pyrimidine biosynthesis, were expressed at significantly higher levels on minimal medium, perhaps reflecting the presence of uridine in the rich medium, which would tend to repress these genes

(47). There are three enzymes involved in conversion of ribonucleotides to deoxyribonucleotides (33, 47), but of the three corresponding genetic loci only the *nrdHIEF* operon was expressed at significantly higher levels on minimal glucose medium. In previous studies, hyperinduction of *nrdEF* by hydroxyurea was measured, but this was the first experiment comparing *nrdEF* transcript levels under normal growth conditions (33), and this was the first indication that the genes encoding the NrdEF accessory proteins, NrdH and NrdI (34), are coregulated with *nrdEF*. There were five genes that were expressed at significantly higher levels in rich medium: the *prsA* gene, which encodes an enzyme that forms the first precursor of purine biosynthesis, and *upp*, *gmk*, *pfs*, and *ndk*, all of which encode enzymes involved in nucleotide salvage or interconversion, consistent with the availability of nucleotides in the rich medium.

Fatty acid biosynthesis and degradation. The *cfa* gene, which encodes an enzyme responsible for postsynthetic formation of cyclopropane fatty acids from unsaturated fatty acids, had a significantly high expression ratio on minimal medium (Table 3; also see Table 6). Since *cfa* is transcribed from an RpoS-dependent promoter (16), this result is consistent with elevated expression of *rpoS* on minimal medium (see below). All of the genes of the *fad* regulon (13) of fatty acid degradation (except for *fadA* [possibly an erroneous result]) were significantly elevated on rich medium, including *fadB*, which is in the *fadAB* operon, and *fadD*, which together encode the fatty acid oxidation multienzyme complex (Fig. 5). Also significantly elevated on rich medium were *fadR*, the repressor of the *fad* genes, and *fadL*, which encodes a long-chain fatty acid transporter. These results tend to indicate that the cells grown on rich medium were exposed to exogenous long-chain fatty acids, leading to induction of the *fad* regulon (14). Interestingly, the *ato* genes, which are involved in degradation of four-carbon fatty acids, were modestly elevated on minimal medium, and the sensor of the two-component regulator of these genes, encoded by *atoS*, was significantly elevated on minimal medium. These results suggest that the cells grown on minimal glucose medium were exposed to acetoacetate, which is the inducer of the *ato* genes (14). *E. coli* is not known to form acetoacetate from glucose, and it is possible that some closely related compound such as acetolactate, which is formed by *E. coli*, serves as an inducer of the *ato* genes (46, 67).

Expression of the genes of fatty acid biosynthesis was generally elevated on rich medium, and, with the exception of *fabB* and *fabG*, all of the *fab* genes were significantly elevated (Fig. 5; Table 3). The relative expression ratios of the genes in the *fabHDG-acpP-fabF* operon corresponded very closely to measurements of transcript levels by Northern analysis (77). In addition, *accA*, which encodes a component of acetyl coenzyme A (acetyl-CoA) carboxylase, was elevated on rich medium. The transcription rate of *accC* is growth rate dependent; the rate is higher in faster-growing cells (16). With the exception of FadR activation of *fabA*, less is known about the regulation of the *fab* genes (16). The data presented here, indicating that the *fab* genes were generally expressed at higher levels on rich medium, suggest that regulation of the phospholipid biosynthesis genes could be growth rate dependent (Fig. 5). This is a reasonable hypothesis, given that faster-growing cells must make membrane components more rapidly. However, the genomic expression data do not prove this hypothesis, and it is also possible that regulation of the *fab* genes is mediated by a signal molecule(s) in the rich medium. Further research in this area will help to clarify the global regulation of phospholipid biosynthesis.

Carbon and energy metabolism. The cells grown on rich medium showed nothing remarkable with respect to the expression pattern of genes involved in carbon and energy metabolism. Of the 409 genes of carbon catabolism, central metabolism, and energy metabolism, only 8 were expressed at significantly higher levels on rich medium (Table 4). These included *nuoM* and *nuoN* of the large operon encoding NADH dehydrogenase I and *cyoA* of the operon encoding cytochrome oxidase *c* (24), suggesting that aerobic respiration was elevated under this growth condition.

Cells grown on minimal glucose medium expressed 31 of the 409 of the carbon and energy metabolism genes at significantly higher levels. These included genes involved in D-lactate utilization (*dld*), acetate formation (*poxB*), regulation of *poxB* expression (*rpoS*), acetate utilization (*aceA*, *aceB*, *gltA*, *icd*, and *mdh*), and coupling of glucose and acetate cometabolism (*uspA*) (Tables 4 and 5). The elevated expression of these genes implicates metabolism of acetate and D-lactate as being perhaps the prominent feature of glucose metabolism in minimal medium. Under this growth condition, cells first consume glucose, which causes repression of the glyoxylate bypass and tricarboxylic acid cycle (15). Simultaneously, the cells excrete acetate and lesser amounts of D-lactate as overflow metabolites (46). As glucose is consumed and acetate accumulates, cells switch smoothly to cometabolism of glucose and acetate (1, 4, 14). This switch involves induction of the tricarboxylic acid cycle and glyoxylate bypass enzymes required to provide energy and to replenish intermediates used for amino acid biosynthesis (14).

Evidence has been published which suggests that pyruvate oxidase (PoxB) forms acetate from pyruvate during the transition from exponential growth to stationary phase: *poxB* expression requires RpoS and thus is elevated during transition phase (11). That cells grown in glucose minimal medium exhibited elevated *poxB* levels supports the contention that acetate was formed via pyruvate oxidation. The elevated expression of *rpoS* during late logarithmic growth (Table 5) also argues that RpoS may play a crucial role in regulating acetate metabolism.

Mutants lacking *uspA* exhibit diauxic growth on minimal glucose medium. This behavior probably occurs because of a failure to assimilate acetate until glucose becomes completely exhausted (49). In the wild-type cells examined here, expression of *uspA* was significantly elevated during growth on glucose minimal medium (Table 5), supporting the argument that UspA somehow plays a critical role in coupling of glucose and acetate cometabolism.

In summary, the evidence presented here provides some insight into the global control of carbon flow in cells growing on glucose in minimal medium. The data argue that acetate overflow metabolism is an important aspect of growth on glucose as the sole carbon and energy source, RpoS may play a role in regulating carbon metabolism genes in late-logarithmic-phase cells, and the universal stress protein, UspA, may coordinate glucose and acetate cometabolism.

Cellular processes and global regulators. Growth on minimal medium with glucose as the sole carbon and energy source places a burden on the cell to synthesize its amino acids de novo or starve. Thus, cells growing on minimal glucose medium are partially starved for amino acids, certainly a stressful situation and potentially having dramatic consequences on global gene regulation, elevating transcript levels of stress-inducible genes, and invoking the stringent response (8, 9, 29, 32). Several of the genes known to be regulated by the stringent-response signal molecule, ppGpp, were found to be differentially regulated on minimal and rich media (Fig. 6; Table

TABLE 4. Genes of carbon and energy metabolism showing significant expression ratios

Functional group	Gene	Gene product	Log ratio (minimal/rich medium)
Catabolism	*amyA*	Cytoplasmic alpha-amylase	0.679
	nanA	*N*-Acetylneuraminate lyase	0.581
	poxB	Pyruvate oxidase	0.535
	galK	Galactokinase	0.519
	ptr	Protease III	−0.629
	clpP	ATP-dependent proteolytic subunit	−0.689
Central metabolism	*gadA*	Glutamate decarboxylase isozyme	1.569
	gadB	Glutamate decarboxylase isozyme	1.497
	aceA	Isocitrate lyase	0.928
	gltD	Glutamate synthase, small subunit	0.889
	aceB	Malate synthase A	0.871
	gltA	Citrate synthase	0.746
	gpmA	Phosphoglyceromutase 1	0.724
	mdh	Malate dehydrogenase	0.587
	rpiB	Ribose 5-phosphate isomerase B	0.580
	phnJ	Phosphonate metabolism	0.538
	icdA	Isocitrate dehydrogenase	0.506
	zwf	Glucose-6-phosphate dehydrogenase	0.503
	nrdE	Ribonucleoside-diphosphate reductase 2, alpha subunit	0.489
	nrdF	Ribonucleoside-diphosphate reductase 2, beta chain	0.478
	tpiA	Triosephosphate isomerase	0.442
	talA	Transaldolase A	0.439
	pfkB	6-Phosphofructokinase II	0.423
	speE	Spermidine synthase	−0.531
Energy metabolism	*nrfC*	Formate-dependent nitrite reductase	0.805
	dld	D-Lactate dehydrogenase	0.726
	nrfA	Periplasmic cytochrome c(552)	0.617
	glpD	*sn*-Glycerol-3-phosphate dehydrogenase (aerobic)	0.616
	nrfB	Formate-dependent nitrite reductase	0.600
	qor	Quinone oxidoreductase	0.497
	ppc	Phosphoenolpyruvate carboxylase	0.477
	atpG	Membrane-bound ATP synthase, F1 sector, gamma subunit	0.451
	nuoJ	NADH dehydrogenase I chain J	0.419
	dsbE	Disulfide oxidoreductase	0.418
	hyfB	Hydrogenase 4 membrane subunit	0.415
	frdD	Fumarate reductase	0.401
	fdnI	Formate dehydrogenase N, cytochrome b_{556} gamma subunit	0.400
	nuoN	NADH dehydrogenase I chain N	−0.412
	ackA	Acetate kinase	−0.446
	cyoA	Cytochrome *o* ubiquinol oxidase subunit II	−0.461
	nuoM	NADH dehydrogenase I chain M	−0.537
	fdoG	Formate dehydrogenase O, major subunit	−0.967

5). Most notable of these genes was *rpoS*, encoding the stationary-phase sigma factor, which was significantly elevated on minimal medium. In fact it appeared that RpoS-dependent gene expression was a prominent feature of the genomic expression pattern of cells grown on minimal medium (Table 6). It is not clear from these genomic expression assays if the elevated level of the *rpoS* transcript was the result of regulation by ppGpp, although this would be consistent with the positive correlation between ppGpp concentration and RpoS levels (9), because it was also found that expression of *nlpD* (which encodes a lipoprotein and is operonic with *rpoS*) was significantly elevated on minimal medium (Table 2). Thus, these data do not distinguish between the possibilities that the higher level of *rpoS* transcription was driven by the *nlpD* promoter or by the *rpoS* promoters located within the upstream *nlpD* gene (29, 38). Production of RpoS is also subject to complex posttranscriptional and translational regulation, and therefore it cannot be presumed that *rpoS* transcript levels are correlated with RpoS activity (29). However, the number of RpoS-inducible genes that were observed to be expressed at significantly elevated levels on minimal medium (21 of them) argues strongly in this case that the *rpoS* transcript level correlated with RpoS function. Interestingly, of the 21 RpoS-dependent genes which were significantly elevated on glucose minimal medium, more than half are known to be involved in the physiological changes that highlight entry into stationary phase (32). However, the cells used in these experiments were in late logarithmic growth phase, still in steady-state growth. Although most studies have focused on the role of RpoS in preparing cells for entry into stationary phase, it has been suggested that RpoS may play a role in logarithmic phase as well (29), and the results presented here support this idea. Since this question is likely to receive further attention, a time course study of genomic expression in cells growing on minimal glucose medium in batch culture would be invaluable.

Some of the regulatory genes had significant expression ratios that were consistent with the elevated transcript levels of their target genes, such as *fadR*, which was expressed at signif-

TABLE 5. Genes involved in cell processes and global regulation showing significant expression ratios

Functional group	Gene	Gene product	Log ratio (minimal/rich medium)
Cell processes	*osmC*	Osmotically inducible protein	1.204
	katE	Catalase	0.893
	msyB	Acidic protein suppresses mutants lacking function of protein export	0.837
	glgS	Glycogen biosynthesis	0.794
	otsB	Trehalose-6-phosphate phophatase	0.777
	fic	Induced in stationary phase, affects cell division	0.654
	glgC	Glucose-1-phosphate adenylyltransferase	0.649
	osmY	Hyperosmotically inducible periplasmic protein	0.580
	bolA	Possible regulator of murein genes	0.564
	otsA	Trehalose-6-phosphate synthase	0.556
	cspD	Cold shock protein	0.540
	cbpA	Curved DNA binding protein	0.540
	envY	Envelope protein, thermoregulation of porin biosynthesis	0.477
	uspA	Universal stress protein	0.469
	motA	Proton conductor component of motor	0.457
	glgB	1,4-α-Glucan branching enzyme	0.430
	sodC	Superoxide dismutase precursor (Cu-Zn)	0.430
	pbpG	Penicillin binding protein 7	0.406
	acrF	Integral transmembrane protein	0.400
	sodB	Superoxide dismutase, iron	−0.904
Cell structure	*nlpD*	Lipoprotein	0.629
	slp	Outer membrane protein induced after carbon starvation	0.549
Regulatory function	*dps*	Global regulator, starvation conditions	1.402
	rpoS	RNA polymerase, sigma S (sigma38) factor	0.766
	atoS	Sensor protein AtoS for response regulator AtoC	0.580
	rseA	Sigma E factor, negative regulatory protein	0.538
	arsR	Transcriptional repressor *ars* operon	0.530
	molR1	Molybdate metabolism regulator	0.521
	lrp	Regulator for leucine (or *lrp*) regulon	0.511
	srlR	Regulator for *gut* (*srl*), glucitol operon	0.464
	tar	Methyl-accepting chemotaxis protein II	0.463
	phoU	Negative regulator for *pho* regulon	0.457
	narP	Nitrate/nitrite response regulator (sensor NarQ)	0.444
	rpoE	Sigma E factor; heat shock and oxidative stress	0.425
	wrbA	*trp* repressor binding protein	0.420
	rcsB	Positive regulator for colanic capsule biosynthesis (sensor, RcsC)	0.416
	fadR	Negative regulator for *fad* regulon, positive activator of *fabA*	−0.662
	cspA	Cold shock protein, transcriptional activator of *hns*	−0.776
	fis	Site-specific DNA inversion stimulation factor; DNA binding protein	−0.818

icantly higher levels on rich medium (as noted above), and *lrp*, which was expressed at significantly higher levels on minimal medium, correlating well with the elevated expression of several genes of the amino acid biosynthetic pathways (48). Several other regulatory genes showing significant expression ratios in this experiment are pleiotropic, and their roles under the growth conditions reported here are not as well understood (Table 5). Among the regulatory genes that were more highly expressed on rich medium are *cspA*, which encodes a cold shock transcription factor, and *fis*, which encodes a factor involved in site-specific recombination and pleiotropic transcriptional regulation (Table 5). Recent evidence indicates that *cspA* is expressed in cells that have not been subjected to cold stress and that its expression is higher in early logarithmic growth phase (7), a pattern of regulation that is remarkably similar to that of *fis* (3, 21, 70), which is also known to be more highly expressed in rich medium (35). Significantly higher in cells grown on minimal medium was *dps*, which encodes a DNA binding protein induced by starvation (42).

What these general DNA binding proteins, Dps and Fis, together with HN-S, seem to have in common is their involvement in growth rate-dependent regulation of gene expression, and it is nearly impossible to discuss these regulators without mentioning RpoS, which either regulates expression of or is regulated by these other factors (8, 9, 29, 32, 35). Together with RpoS, HN-S-dependent gene expression was prominent in the genomic transcription pattern of cells grown on minimal medium, and in fact the four genes with the highest expression ratios on minimal medium, *hdeA*, *hdeB*, *gadA*, and *gadB* (*dps* was fifth highest [Tables 4 and 6]) are known to be regulated by HN-S (5, 74, 75). Interestingly, *hns* expression was similar in minimal and rich media, suggesting either that expression of the *gad* and *hde* genes was not regulated by HN-S under these conditions or that *hns* expression (or HN-S function) is subject to posttranscriptional regulation by a mechanism which has yet to be described. In fact, expression of *gadB* and *hdeAB* in *Shigella flexneri* (72) and also *gadA* and *gadB* in *E. coli* (10) is RpoS dependent.

The apparent connection between these HN-S-regulated genes is their involvement in acid resistance (10). The unlinked genes, *gadA* and *gadB*, encoding homologous glutamate decarboxylases (62), are thought to be induced during fermentation as a result of acid stress (5, 61, 72). The *gadB* gene appears to form an operon with *xasA* (*gadC*) in *E. coli* (10) and is known

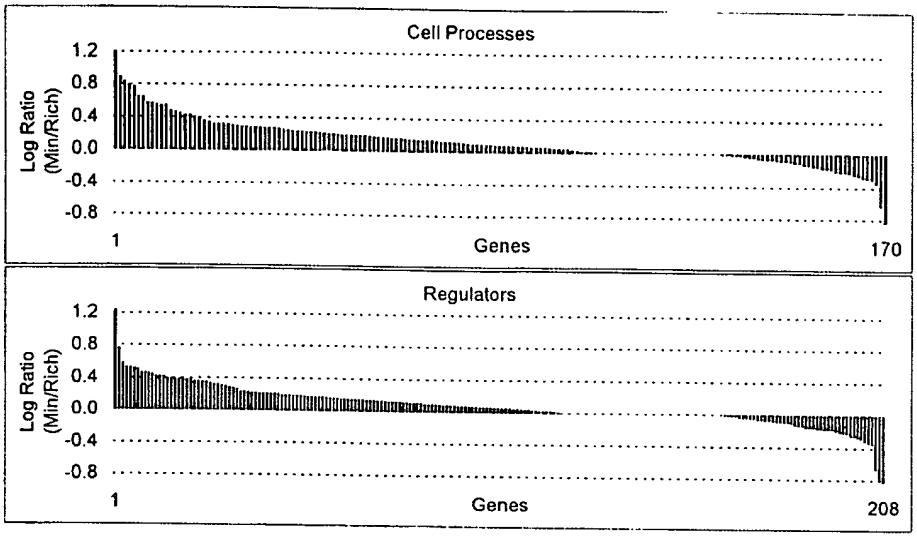

FIG. 6. Log expression ratios of cell process genes and regulatory genes sorted by value for the minimal glucose versus Luria broth plus glucose experiment.

to be cotranscribed with *xasA* (*gadC*) in *S. flexneri* (72); *gadC* mutants of *E. coli* are acid sensitive (30). Not surprisingly, *xasA* (*gadC*) had a significant log expression ratio on minimal medium (0.580 [data not shown]). Clustered together with *gadA* and *hdeAB* are several other genes that showed significantly higher expression ratios on minimal medium, i.e., *hdeD*, *yhiE*, and *yhiX* (log expression ratios of 0.872, 0.852, and 1.096, respectively [data not shown]). The functions of these five genes are all unknown, but it has been shown that *hdeAB* mutants of *S. flexneri* are acid sensitive (72). The *yhiX* gene, which encodes an AraC-like protein, is a likely candidate for regulation of the *gad* and *hde* genes, given its position downstream of *gadA* and its high expression ratio on minimal medium. Furthermore, alignment of the *gadA*, *gadB*, and *hdeD-hdeAB* regulatory regions (200 bp upstream of start codons) revealed a 19-bp sequence which is perfectly conserved in *gadA* and *gadB* and of which 15 bp are conserved in all three (data not shown). In summary, the results suggest that these HN-S/RpoS-dependent genes comprise a system for acid tolerance. It is interesting to speculate that RpoS plays a role in these logarithmic-phase cells of coordinating induction of the acid tolerance genes, together with the genes of organic acid me-

TABLE 6. Genes regulated by RpoS showing significant expression ratios

Functional group	Gene	Gene product	Log ratio (minimal/rich medium)
Catabolism	*galK*	Galactokinase	0.519
	poxB	Pyruvate oxidase	0.535
Cell processes	*bolA*	Possible regulator of murein genes	0.564
	cbpA	Curved DNA binding protein	0.540
	fic	Induced in stationary phase, affects cell division	0.654
	glgS	Glycogen biosynthesis	0.794
	glgC	Glucose-1-phosphate adenylyltransferase	0.649
	glgB	1,4-α-Glucan branching enzyme	0.430
	katE	Catalase	0.893
	osmC	Osmotically inducible protein	1.204
	osmY	Hyperosmotically inducible periplasmic protein	0.580
	otsB	Trehalose-6-phosphate phophatase	0.777
	otsA	Trehalose-6-phosphate synthase	0.556
Energy metabolism	*frdD*	Fumarate reductase, anaerobic	0.401
	glpD	*sn*-Glycerol-3-phosphate dehydrogenase	0.616
FA and PL metabolism	*cfa*	Cyclopropane fatty acyl phospholipid synthase	0.618
Hypothetical	*hdeA*	ORF, hypothetical protein	1.872
	hdeB	ORF, hypothetical protein	1.705
Regulatory function	*dps*	Global regulator, starvation	1.402
	rpoS	Sigma S (sigma 38) factor	0.766
	wrbA	*trp* repressor binding protein	0.420

tabolism, under conditions of glucose overflow metabolite formation.

Conclusion. In the single experiment presented here, the hallmark features of growth on minimal and rich media were revealed. Across the genome, we observed differences in the expression of functionally grouped genes that paralleled the physiology of these two growth conditions. Cells grown in rich medium with a good carbon and energy source, glucose, grew rapidly, turning off the pathways of biosynthesis and elevating the expression of the genes involved in macromolecule synthesis, most prominently protein synthesis. Cells in minimal medium faced the need to synthesize all of their building blocks from a single carbon and energy source, again glucose, and this burden was reflected not just in the turning on of biosynthetic pathways but also in the elevated expression of regulators of cell processes and regulons involved in stress tolerance. The most prominent features of growth on glucose minimal medium were the formation of overflow metabolites, in particular acetate, and protection of the cell from the stress of living in a self-formed acidic environment. All of these aspects of physiology were revealed not by painstaking and careful analysis in the laboratory of each system but, rather, by deduction from the genomic expression patterns of cells grown under these two rather different conditions. These deductions would not have been possible were it not for countless microbial physiology experiments published over the past 50 years (44). On the other hand, from the one simple experiment reported here, the tremendous potential of functional genomics is obvious. As a result of this experiment, several genes were added to functional groups on the basis of coregulation with similar and related genes. Also, several testable hypotheses were generated, in particular those involving the flow of carbon to acetate, coupling of glucose and acetate cometabolism, and acid resistance, the importance of which has been previously pointed to in enteric bacteria (4).

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